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Quantitative aspects of *Mycoplasma pneumoniae*-cell relationships in cultures of lung diploid fibroblasts

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INTRODUCTION

This report describes quantitative studies on the growth of *Mycoplasma pneumoniae* in cultures of the WI-38 diploid cell strain. Several aspects of this were investigated, and as a result an interesting model of host-parasite relationship emerged. The feasibility of extending this model to the quantitative assay of antibiotics is discussed. According to recent observations the inhibitory effect of antibiotics may be strongly affected by the milieu in which mycoplasmas are perhaps protected by their close association with cells (Gori & Lee, 1964; Friend, Patuleia & Nelson, 1966). That cell attachment involves an active biological process on behalf of either cell or mycoplasma has recently been reported by Fogh & Fogh (1967), who have also convincingly demonstrated that quantitative models of mycoplasma-cell interactions may lend themselves to more meaningful studies of effects of antibiotics.

The significance of the capacity of some mycoplasmas to reside in the cytoplasm was first recognized by Hayflick & Stinebring (1955). This provided impetus for further investigations of mycoplasmas in cell culture (Hayflick, Stinebring, Breckenridge & Pomerat, 1956; Hayflick, 1956; Hayflick & Stinebring, 1960). Morphological studies of *M. pneumoniae* in experimentally infected monkey kidney cells were made by Clyde (1961).

Subsequently, mycoplasmas were frequently found as contaminants in a variety of cell cultures. The possible source of this type of contamination, the problems involved and the means by which cell cultures could be decontaminated were reviewed by Hayflick (1965*b*), Hayflick & Chanock (1965) and MacPherson (1966).

The experimental results to be described below are each based upon six separate assay dates.

MATERIALS AND METHODS

Mycoplasma

The FH strain of *M. pneumoniae*, kindly provided by Dr L. Hayflick, was used in these studies. This strain had undergone 156 consecutive passages in acellular media. Before these experiments, it had not been passaged in cell cultures.

Cell cultures

Cell monolayers of the WI-38 diploid cell strain were prepared in 4 in $\times \frac{1}{2}$ in tubes by the standard methods described by Hayflick (1965*a*).

Acellular media

The media used (PPLO broth and agar) were those described by Chanock, Hayflick & Barile (1962). These media contained beef heart infusion base (Difco) and fresh yeast extract (Hers) as described by Lemcke (1965).

Inoculation of cell cultures

Inocula. These were prepared by diluting with SM 199 a 3-day-old mycoplasma culture in the liquid acellular medium. The SM 199 contained no serum in order to avoid inhibitors. Serial tenfold dilutions of the inocula thus prepared were seeded on agar plates to determine retrospectively the number of viable organisms in the inocula.

Procedures. The growth medium was removed from confluent cell monolayers and replaced with 1 ml. mycoplasma inoculum. Inoculated cell cultures were incubated at 36°C. for periods up to 6 days.

Multiplicity of infection

In these quantitative studies it was important to be able to define the multiplicity of infection (i.e. the number of mycoplasmas in the inoculum per host cell). The average number of cells of the WI–38 strain grown as confluent monolayers in tubes was calculated to be approximately 5×10^5 by a method described by Larin & Roberts (1963). From this figure, the multiplicity of infection was calculated for each mycoplasma inoculum.

Mycoplasma recovery and enumeration from cell cultures and from broth

The number of viable mycoplasmas, cell-associated or extra-cellular, was determined for each incubation day. The supernatant fluid from four inoculated cell monolayers was pooled and the cells were washed three times using 1 ml. of SM 199 per tube for each wash. The washed cell monolayers were removed with a rubber scraper and the cells were resuspended in 4 ml. of fresh SM 199, in which they were disintegrated using an MSE homogenizer to liberate the cell-associated mycoplasmas.

Serial decimal dilutions of the cell culture supernatant and the cell homogenate were made separately in SM 199. Similar dilutions of PPLO broth cultures were made in PPLO broth. Viable counts were made by the method of Miles & Misra (1938), modified by using one drop of 0·05 ml. per plate, which was spread over the surface of the agar with a platinum loop. If the count was expected to be low, duplicate plates were each inoculated with three drops of 0·02 ml., which were not spread with a loop but left to form circular patches of about 20 mm. diameter. All plates were incubated for 10 days at 36°C, in closed chambers to prevent further drying of the medium.

Colonies were counted at a magnification of × 25, using a Zoom stereoscopic microscope (W. Watson & Sons, Ltd, London).

The reliability of the colony counting method was examined for conformity with a Poisson distribution and was found to be satisfactory. The colony counts of

consecutive decimal dilutions of mycoplasma cultures were compared and found to be very near to the expected decimal relationship.

Microscopical demonstration of mycoplasma effects on cell cultures

Cultures of infected cells on coverslips were washed three times in phosphate buffered saline, pH 7·3, to remove extracellular mycoplasmas, and then the cells were stained with orcein by a similar method to that of Fogh & Fogh (1964). The coverslips were examined under the microscope at magnifications from $\times 400$ to $\times 3200$.

Wherever other materials or techniques have been used, they are described in the text below.

RESULTS

The survival of mycoplasmas in SM 199 *and in SM* 199 + *WI–38 cell extract*

A mycoplasma inoculum sufficient to give an initial colony count of ca. 10^4 viable particles/ml. in liquid media was thought desirable for this study in order to allow conditions of maximal survival. For this purpose a mycoplasma culture in PPLO broth which contained ca. 10^6 viable particles/ml. was diluted 1/100 in PPLO broth, SM 199 or SM 199 + WI–38 cell extract and these media were incubated at 36° for 7 days. As shown in Table 1, the survival of *M. pneumoniae* in SM 199 alone or in SM 199 + WI–38 cell extract was very poor.

Table 1. *Growth of* Mycoplasma pneumoniae *in PPLO broth, and in SM 199 with and without WI–38 cell extract*

	Viable count (CFU/ml.) in		
Day	PPLO broth	SM 199	SM 199 with WI–38 cell extract
2	81,100	120 (0·86)	80 (0·57)
4	5,760,000	0	0
7	1,455,000	0	0

Inoculum, $1{\cdot}4 \times 10^4$ CFU/ml. The figures in parentheses are percentages of the original inoculum

With smaller inocula no mycoplasmas were recovered from SM 199 or SM 199 + WI–38 cell extract 24 hr. post inoculation. As a result of these experiments it is concluded that with massive inocula as above a small number of organisms may survive for 48 hr. in SM 199 due to materials carried over from the PPLO broth.

WI–38 cell-associated mycoplasmas in relation to extracellular mycoplasmas and to the multiplicity of infection

As shown in Table 2 and Fig. 1, the amount and the time of appearance of cell-associated and extracellular mycoplasmas were related to the number of CFU/ml. in the inoculum and thus to the multiplicity of cell infection. With a relatively high multiplicity, such as 0·095 ($10^{4{\cdot}7}$ CFU/ml. inoculum), no apparent lag phase occurred, although this could not be excluded, and the amounts of cell-associated and extracellular mycoplasmas were similar on each day of the 6-day experimental period.

At lower multiplicity of infection, there followed a definite lag phase when no mycoplasmas, cell-associated or extracellular, were detectable. Following the lag phase, the cell-associated mycoplasmas were always the first to appear and the extracellular mycoplasmas could not be detected till 1 day later. The lag phase became longer as the multiplicity of infection decreased (Table 2). The growth from the zero point after the lag phase was extremely rapid with the average generation time of 2·72 hr. (cell-associated: 2·71–2·79; extracellular: 2·65–2·75). There was also a linear relationship between increases of the cell-associated and extracellular mycoplasmas (Fig. 1). This finding is what could be expected on the hypothesis of the extracellular mycoplasmas being derived from cell-associated replication.

Table 2. *The amount and time of appearance of cell-associated and extracellular mycoplasmas in infected cell cultures*

	Multiplicity of infection							
	0·095		0·0079		0·00052		0·00007	
	Inoculum concentration*							
	4·7 (47·600) Mycoplasma count*		3·6 (3·930) Mycoplasma count*		2·4 (260) Mycoplasma count*		1·5 (35) Mycoplasma count*	
Days after inoculation	Cell-associated	Extracellular	Cell-associated	Extracellular	Cell-associated	Extracellular	Cell-asosciated	Extracellular
1	5·0 (99,900)	4·9 (88,700)	0	0	0	0	0	0
2	5·9 (856,000)	5·7 (529,000)	0	0	0	0	0	0
3	6·7 (4,860,000)	6·4 (2,720,000)	2·4 (260)	0	0	0	0	0
4	6·8 (5,960,000)	6·7 (5,160,000)	5·3 (221,000)	4·1 (12,600)	1·9 (80)	0	0	0
5	6·5 (3,160,000)	6·6 (4,300,000)	6·3 (2,060,000)	5·5 (286,000)	5·2 (150,600)	4·0 (9,900)	0	0
6	6·2 (1,596,000)	6·3 (1,956,000)	6·7 (4,880,000)	6·5 (2,860,000)	6·3 (1,806,000)	5·3 (202,000)	1·0 (10)	0

* $\log_{10}$ CFU/ml. with numbers of CFU/ml. in parentheses.

In summary, the data in Table 2 and Fig. 1 appear to fit a conclusion that cell infection of a relatively high multiplicity, such as 0·095, results in saturation of the cell monolayer, most likely explained by rate of cell attachment, with excess mycoplasmas passing freely to the supernatant fluid. At lower multiplicity, all mycoplasmas in the inoculum attach to the cells and there follows a lag period of 2 to 5 days during which infection is cell-associated. The occult period, during which the micro-organism is not recoverable either from the supernatant or from the cells, is a phenomenon of some considerable theoretical and practical interest.

Table 2 and Fig. 1 show that, though mycoplasmas were recoverable from an inoculum containing as little as $10^{1·5}$ CFU/ml., none of a 100 times greater amount of mycoplasmas, presumably present in a homogenate of cells infected with the $10^{3·6}$ CFU/ml. inoculum, could be recovered from it on agar plates during the first

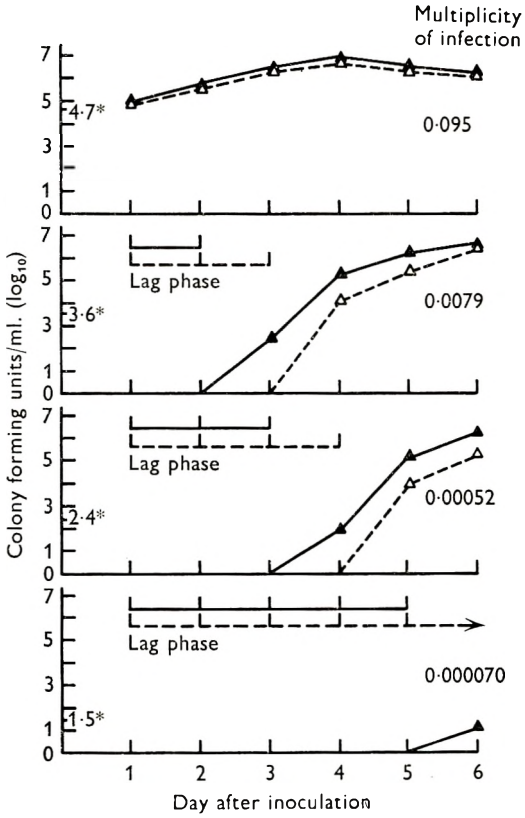

Fig. 1. The amount and time of appearance of cell-associated and extracellular mycoplasma in infected cell cultures. ▲—▲, Cell associated; △- -△, extracellular. * inoculum concentration.

Table 3. *Average yields of mycoplasmas (Log_{10} CFU/ml.) from cell culture and acellular medium*

Time after inoculation		Cell culture*			Acellular medium†
Days	Hours	Cell-associated	Extracellular	Total	
1	8	—	—	—	4·62
	24	5·18	5·23	5·50	5·00
	32	—	—	—	5·58
2	48	6·40	6·38	6·69	5·90
	56	—	—	—	6·11
3	72	6·64	6·61	6·92	6·27
	78	—	—	—	6·25
4	—	6·57	6·60	6·88	‡
5	—	6·38	6·39	6·68	—

* Inoculum concentration = $10^{4·52}$ CFU/ml., multiplicity of infection = 0·07
† Inoculum concentration = $10^{4·55}$ CFU/ml.
‡ Experiment discontinued.

2 days after cell infection, and on day 3 the cells only yielded approximately 7% of the mycoplasma content of the inoculum. After day 3, the amount of mycoplasmas in cell homogenates increased logarithmically, and they appeared also in the supernatant fluid.

Generation time in cell culture and acellular medium

The results of our experiments to compare the growth of *M. pneumoniae* in cell culture and in acellular medium which was inoculated with similar amounts of mycoplasmas ($10^{4\cdot52}$–$10^{4\cdot55}$) are shown in Table 3 and Fig. 2. The growth curve in

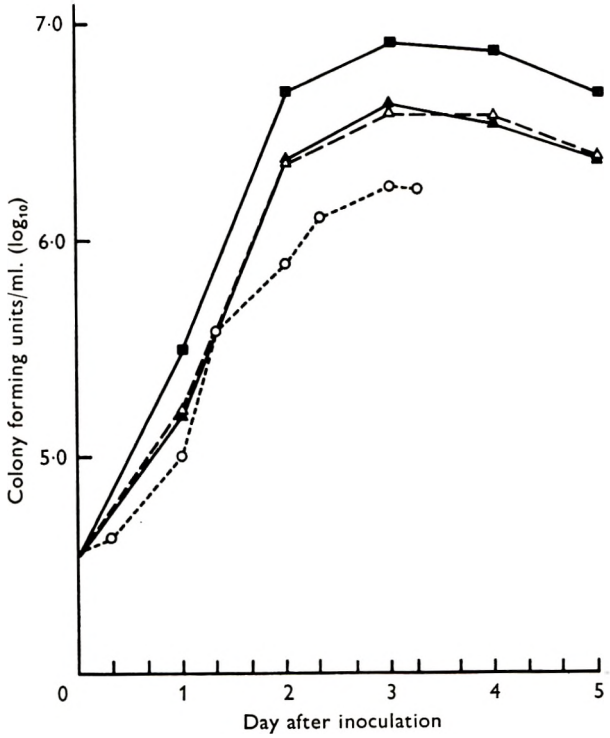

Fig. 2. Average yields of mycoplasma (Log_{10} CFU/ml.) from cell cultures and acellular medium. ▲—▲, cell-associated; △ – – △, extracellular; ■—■, cell-associated and extracellular; ○ – – ○, in acellular medium.

both cultures followed a similar pattern but a higher yield of the micro-organism was obtained in the cell culture. This shows that mycoplasma may prefer the cellular environment rather than acellular medium for their propagation.

Generation times during the logarithmic phase of growth were calculated and found to be as follows. For cell-associated and extracellular mycoplasmas, i.e. for the organism recovered from either the cell homogenate or the supernatant, the generation time was very similar, i.e. 6·1 hr. for each, and for the total from the cell homogenate and the supernatant fluid it was 6·0 hr. In ten similar experiments with inocula ranging from 4·36 to 5·21 $\log_{10}$ CFU/ml., the average generation time was

6·5 hr. for the cell-associated mycoplasmas and 6·2 hr. for the total of cell-associated and extracellular mycoplasmas (Table 4).

With smaller inocula, i.e. $10^{3\cdot6}$ to $10^{1\cdot5}$, which produced in infected cell cultures a lag phase during which no mycoplasma could be isolated for a period of 2 to 5 days (Table 2 and Fig. 1), the subsequent multiplication was extremely rapid with the average generation time of 2·72 hr. In contrast, the generation time in the acellular medium was 8·0 hr. Low & Eaton (1965) obtained average doubling times of 5 to 12 hr. in acellular medium. They found that the results obtained were influenced by the number of passages the organism had experienced. The FH strain of *M. pneumoniae* which was used in our experiments had undergone 156 passages in acellular media, but it had not previously been grown in cell culture. Therefore, the shorter generation time with this strain when grown in cell culture suggests more favourable conditions for mycoplasma propagation in association with the mammalian cell.

Table 4. *Average generation times for M. pneumoniae in cell culture*

Expt. no.	Inoculum concentration ($\log_{10}$CFU/ml.)	Multiplicity of infection	Mycoplasma generation time (h)	
			Cell-associated	Total cell-associated and extracellular
1	4·78	0·05–0·18	7·20	6·87
2	5·21		7·03	6·15
3	4·74		6·53	6·00
4	4·96		4·70	4·20
5	4·77		6·20	6·20
6	4·45		6·68	7·05
7	4·93		7·05	7·05
8	4·95		7·10	7·16
9	4·60		6·31	6·31
10	4·36		6·48	5·00
Average generation time			6·53	6·20

Effects of pH changes

Over the 6-day period of the experimental observations the pH values of the supernatant fluid in infected cell cultures was falling steadily from 7·1 or 7·2 to between 6·3–6·7 by the sixth day. The fall of the pH was greater in cell cultures infected with concentrated inocula, i.e. of the order of $10^{4\cdot5}$/ml. to $10^{5\cdot1}$/ml. mycoplasmas, but it was also observed when smaller inocula were used.

It was reported that with *M. pneumoniae* in acellular media there was a rapid loss of viability of the micro-organism once the pH value fell below 6·5 (Low & Eaton, 1965) and that no growth occurred at pH 6·0 (Shepard & Lunceford, 1965). In our studies a pH of 6·3 to 6·7 in the supernatant in infected cell cultures by the sixth day after inoculation has not affected the viability of cell-associated and extracellular mycoplasmas. This suggests that the organism in cell association is protected from the alkali-acid pH change in the supernatant fluid.

Morphological aspects

Examination of the stained preparations of the WI–38 cell cultures infected with mycoplasma showed that the mycoplasmas were located on the cell surface and probably inside the cells. The number of cell-associated mycoplasmas varied with the multiplicity of infection and with time of inoculation from few individual micro-organisms to heavy confluent masses in the cytoplasm and in the intercellular spaces. Disintegration of infected cell monolayers also varied with the multiplicity of infection and with time of incubation. On some occasions the whole of the cell monolayers would disintegrate by the seventh day but on other occasions it remained completely intact even after infection of a high multiplicity.

The infected cells showed morphological changes on the first examination, i.e. as early as 24 hr., with the appearance in a few cells of slight granulation in the cytoplasm; by 48 hr. more definite granulation appeared, mainly at the cell border and cellular processes. After the fourth day of infection large areas of the cell monolayer showed granulation of the cytoplasm and loss of cells was beginning to appear. The cell destruction never occurred before the maximum level of mycoplasma growth had been reached, i.e. day 4 to 5 after infection. To conclude, the morphological changes in our experiments were in all essentials similar to those observed by Fogh & Fogh (1964, 1967) with different mycoplasma-cell systems.

DISCUSSION

It has been known for a long time that mycoplasmas may be closely attached to the living cells so that they cannot be separated from them. Though discussed widely, the problem of the mode of mycoplasma-cell association has not yet been resolved. It may be that mycoplasmas may grow only on the surface of cells and in the intercellular spaces and their appearance in the cytoplasm may be the result of pinocytosis. Alternatively, they may be capable of intracellular residence in which they may assume a stage that allows survival only in association with the host cell. A clear understanding of the mode of mycoplasma-cell association will obviously have to await better knowledge of this group of microbes.

The main purpose of this study was to investigate the quantitative aspects of the growth of cell-associated and extracellular forms of *M. pneumoniae* in cultures of lung diploid fibroblasts as represented by the WI–38 cell strain. With this aim in mind we purposely selected a well-known laboratory strain (FH) of *M. pneumoniae* with a history of many passages in acellular media to attempt to answer the question whether or not these have reduced the original capacity of the micro-organism to invade living cells. The fact that this strain was never, to the best of our knowledge, passaged in cell cultures was a further inducement in these studies.

As reported in this paper, the WI–38 cell strain was shown to be a sensitive, susceptible host for the FH mycoplasma strain. The pattern of mycoplasma replication in cell cultures was quite specific in all experiments and the detectability and increases of cell-associated and extracellular mycoplasmas appeared to be closely related to the multiplicity of cell infection. At relatively high multiplicity levels

there was no substantial quantitative difference between the cell-associated and extracellular mycoplasmas in regard to the amount and the time of appearance. In contrast, with lower multiplicity it was found that the cell-associated mycoplasmas were preponderant and at the multiplicity of cell infection as low as 0·00007, i.e. 1 mycoplasma per 14,000 cells, only the cell-associated mycoplasmas were detectable and then only after a lag period of 5 days.

The puzzling problem of the occult period which occurs in cell infections of lower multiplicity deserves further study. It seems that during this period mycoplasmas cannot be recovered from infected cell cultures using the acellular medium, in which they have been propagated before their contact with living cells. This may be explained in various ways. It may be that the attachment to cell receptors (Taylor-Robinson & Manchee, 1967) and the process of cell pinocytosis weakened mycoplasmas in the homogenate and they temporarily lost the capacity to divide in acellular medium. Alternatively, they may have temporarily assumed a stage which allows survival only in association with living cells (Klieneberger-Nobel, 1962). That cell attachment involves an active biological process on behalf of either cell or mycoplasma has recently been convincingly demonstrated with different mycoplasma-cell systems (Fogh & Fogh, 1967; Taylor-Robinson & Manchee, 1967).

On the present evidence it seems possible that the occult period observed in our experiments (Table 1, Fig. 1) may have been due to a specific association between mycoplasmas and infected cells in culture. The possibility that the cells have provided only a convenient inert surface seems unlikely; if this would be the case, then mycoplasmas should have been recovered from cells infected at low multiplicity levels (Table 1) in the way they have been recovered from the inocula, i.e. without any occult period.

The origin of the increasing extracellular mycoplasmas in infected cell cultures is a difficult but also a most interesting question. It is quite certain that SM 199 alone or after supplementation with the WI–38 cell extract does not support the growth of *M. pneumoniae*. It cannot be ruled out, of course, that the infected cell cultures may produce some substances which enable mycoplasmas to grow in the SM 199 supernatant. On the other hand, it is clear from our experiments with lower multiplicity of infection that evidence for such assumption is lacking. It is obvious, therefore, that most extracellular organisms were produced in association with infected cells, being liberated into the supernatant from the partly destroyed cytoplasm.

It must be pointed out that this investigation was limited to one strain of mycoplasma and one type of cell culture, and it is quite possible that other strains may show different patterns of their growth in different cell cultures. Thus each strain may pose a separate problem. A further problem is the sensitivity of the method using acellular media for recovery from infected cells of mycoplasmas which have temporarily assumed a stage which allows survival only in association with living cells.

Although the mode of mycoplasma-cell interaction has not yet been settled, it is already clear that mycoplasmas are not easy to kill in cell environment without also killing the cell. With regard to eradication of mycoplasmas from contaminated cell cultures, the most recent reviews indicate that, regardless of the source of the

initial contamination, there is no effective decontamination method which is universally applicable (Hayflick & Chanock, 1965; MacPherson, 1966). Some drastic methods which were reported as successful for decontamination of cell cultures could not of course be used for eradication of mycoplasmas in the body.

That antibiotics can penetrate mammalian cells is clearly shown in a review by Collins (1965). Much less is known, however, of the probability of a circulating antibiotic crossing a number of barriers that separate it from an intracellular parasite (Kessel, 1965). It appears, then, that caution has to be exercised in the determination of the potency of antibiotics against mycoplasmas by methods employing acellular media alone, and it is desirable to utilize also appropriate cell culture models. A possible approach to designing such models is provided by this study.

SUMMARY

The FH strain of *Mycoplasma pneumoniae* was studied in experimentally infected cultures of lung diploid fibroblasts. The multiplicity of infection was found to have a profound influence on the distribution of the cell-associated and extracellular mycoplasmas. At a high multiplicity there was very little difference between the ratio of cell-associated and extracellular mycoplasmas. In contrast, with a low multiplicity the cell-associated mycoplasmas predominated and with further reductions of the inoculum only the cell-associated mycoplasmas could be detected. The significance of these findings is discussed. It is suggested that caution has to be exercised in the determination of the potency of antibiotics against mycoplasma by methods employing acellular media alone, and it is desirable to utilize also appropriate cell culture models. A possible approach to designing such models is provided by this study.

We express our appreciation to the Tissue Culture Laboratory, Pfizer Limited, Sandwich, for kindly supplying us with cell cultures. We furthermore thank the Board of Directors, Pfizer Limited, for permission to publish this study. The helpful discussions with our colleagues Drs D. Jackson and G. M. Williamson in preparing the manuscript are acknowledged with thanks.

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With 1 *plate*
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A cyclone separator for aerosol sampling in the field

By F. P. ERRINGTON and E. O. POWELL
Microbiological Research Establishment, Porton Down, Salisbury, Wilts.

(*Received* 11 *October* 1968)

INTRODUCTION

The concentration of bacteria in the open air is usually very small—of the order of 1 viable organism in some tens of litres. Spores and fragments of moulds are more frequent by a factor of about 100. Both figures fluctuate wildly with season and weather, and even from hour to hour. Higher concentrations are found downwind of local sources of contamination; for example, ploughing and harrowing in dry soil and discharge of raw sewage. But even in such instances nearly all the organisms which become airborne are of no consequence to public health or agriculture except perhaps as indicators. Effective biological analysis of the airborne particles, and especially the detection of significant changes in concentration, therefore calls for the use of samplers working at a high flow rate and capable of concentrating the particulate contents of a large volume of air into a small volume of liquid.

Large cyclones have long been in industrial use as coarse filters for removing dust. But to be effective in removing fine particles a cyclone must be small in physical size and must be worked at a high gas velocity. No satisfactory theory of the cyclone has been evolved, but existing analyses suggested that a cyclone of the dimensions shown in Fig. 1 would (*a*) collect 50% of particles 0·85 μ in diameter (Lapple, 1950), or (*b*) collect all particles greater than 2·2 μ in diameter (Davies, 1952), the particles being of unit density and the flow rate 75 l./min. Fuchs's (1964) general discussion further supported this estimate, on which our design is based.

A cyclone which is to be used as a sampling device and not merely as a scavenger must be provided with a ready means for removing the deposited particles either continuously or at frequent intervals. In large cyclones cleaning is often carried out by injecting a spray of water (Stairmand, 1956). The water drops are themselves thrown to the walls and wash down the deposit through the tail pipe into an air-tight container. Whether the same principle would be effective on a small scale seemed at first doubtful, if only because of surface tension effects. Experiment showed that injected water collected as an uneven film completely covering the inner wall and moving helically towards the lower apex, where the rotation was very rapid and turbulent. Continuous scrubbing was achieved by inducing a slight suction at the tail pipe, so that the liquid was drawn into the receiver.

CONSTRUCTION AND USE

The cyclone has been made in two sizes in either Perspex or stainless steel (Plate 1). Stainless steel is much the heavier and more difficult to machine, but its robustness is a great advantage in field work, where the operator's care may be reduced

by exposure to uncomfortable conditions for long periods. The smaller cyclone is intended to work at a flow rate of about 75 l./min. with a pressure drop of 75 mm. Hg; the larger at about 350 l./min. at a pressure drop of 200 mm. Hg. It will be sufficient to describe the smaller.

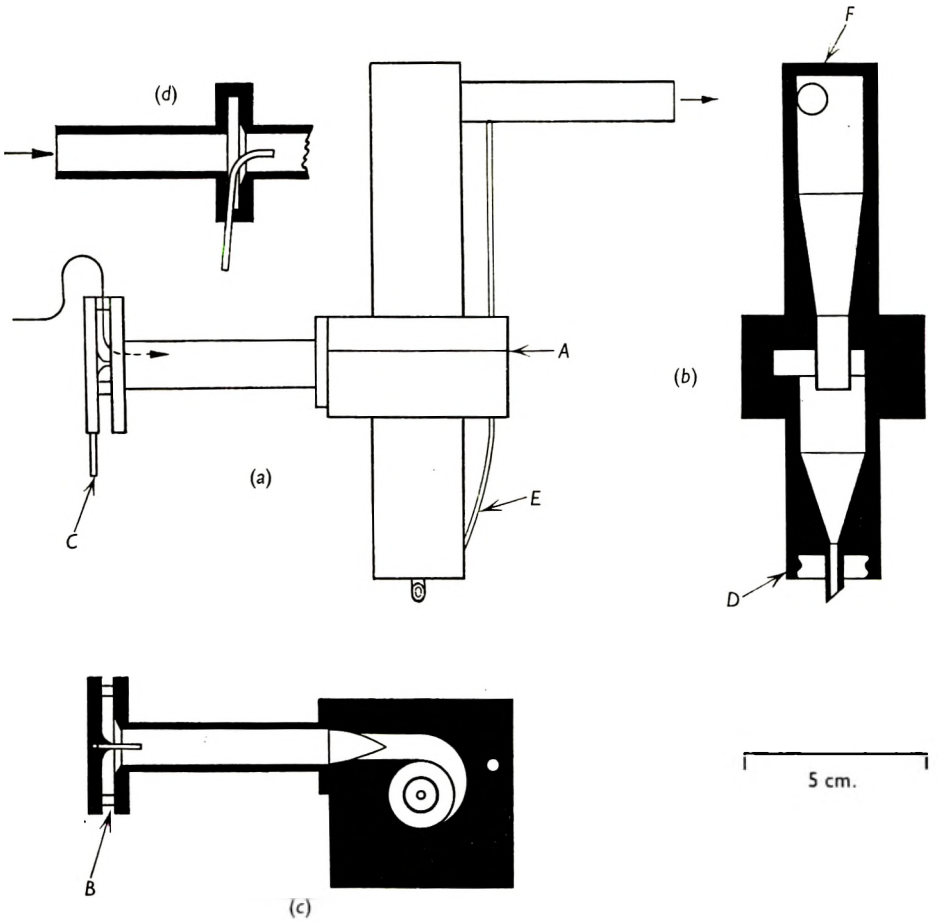

Fig. 1. (*a*) Side elevation of cyclone. *A*, Junction of upper and lower components; *C*, side-tube for introducing scrubbing liquid; *E*, tube connecting receiver to outlet. (*b*) Axial section normal to axes of inlet and outlet pipes. *D*, Hood for connecting sample receiver, *F*, outlet pipe set tangentially. (*c*) Transverse section through axis of inlet pipe. *B*, Annular gap for entry of aerosol. (*d*) Adapter for coupling to closed chambers; section.

The internal diameter over the cylindrical part (Fig. 1*b*) is $\frac{1}{2}$ in., and the other dimensions are in the proportions suggested by Lapple (1950), except that there is a volute entry (Fig. 1*c*). The throat is $\frac{1}{4}$ in. square in section, giving an average normal acceleration of about 10^7 cm./sec.^{2} ($10^4 \times$ gravity) at the top of the cyclone. The main body is constructed in two parts, joined at *A* (Fig. 1*a*). Before assembly the volute is formed by end-milling in the upper face of the lower part, and the overflow pipe by machining the lower face of the upper part. Thereafter the inlet tube is

screwed on and the junction faired from a circular to a square section by means of a taper reamer. The actual inlet is an annular gap between two plates (*B*; Fig. 1*c*), one of which is a flange on the inlet tube. The other carries a fine tube at its centre through which the scrubbing liquid is fed *via* the side-tube *C* (Fig. 1*a*). In field use, the face of the outer plate is presented to windward and serves to exclude coarse particles. This plate may be replaced by an adapter (Fig. 1*d*) for sampling from experimental chambers.

Samples are collected in bijou bottles screwed into the hood *D* (Fig. 1*b*) enclosing the tail pipe; the joint must be airtight. In order to ensure the transfer of the scrubbing liquid to the bottle, the hood is connected by a fine tube, 0·6 mm. inside diam. (*E*; Fig. 1*a*) to the outlet, where the pressure is lower. Even in the absence of liquid the air flow ('underflow') through the side-tube is very small compared with the total flow, and cannot appreciably upset the working. The scrubbing liquid is best supplied by a peristaltic pump giving a continuous flow. The pumping rate should be variable within the range 0·5–2 ml./min., because only a variable fraction of the injected liquid reaches the receiver. There are two causes of loss: (i) Some of the liquid impinges in the volute, and the reverse eddy in this region (van Tongeren, 1935) causes part of this liquid to creep over the roof of the cyclone and the outside of the overflow tube into the outlet. The loss of deposited particulates (most of which impinge at a lower level) from this cause appears to be negligible; (ii) The scrubbing liquid is brought into intimate and violent contact with the air; the amount which evaporates varies with the ambient humidity. The pump, then, should be adjusted so that liquid is delivered to the receiver at an approximately constant rate, e.g. about 0·75 ml./min.; the particulate contents of 100 l. of air are then concentrated into about 1 ml. of liquid. The sample volumes should be measured, or else made up to a convenient fixed volume.

In order to minimize pressure drop and at the same time to encourage entrained liquid to creep along the walls rather than to be broken up and become airborne, the overflow tube widens gradually into a cylindrical enlargement in which the outlet tube is set tangentially (*F*; Fig. 1*b*). Most of the entrained liquid evaporates in this upper chamber.

The air flow through the cyclone is best regulated by interposing a critical orifice (Druett, 1955) between cyclone and pump. The orifice should be rated to pass 84 l./min.; this corresponds to a flow of 75 l./min. of free air entering the cyclone. The pump displacement then needs to be 120–150 l./min.; a suitable pump is the 'Wade Minor 300F' (Messrs. J. T. Wade and Sons, Ltd., High Wycombe, Bucks).

The linear dimensions of the larger cyclone are twice those of the smaller (Plate 1), and the proportions are the same except that the throat is relatively smaller; it is rectangular, $\frac{1}{2}$ in. deep in the direction of the main axis and $\frac{3}{8}$ in. wide in the radial direction. The larger cyclone permits of concentrating the particulate contents of 500 l. of air into 1 ml. of liquid. The loss by evaporation of the liquid is correspondingly greater.

The pressure at the lower apex of a cyclone is below that at the inlet, and no liquid is lost if a sample container is detached. At the same time little liquid can accumulate during the few seconds required to change a container, and it is un-

necessary to interrupt the flow of either liquid or air. It is thus easy to obtain an indefinitely long and uninterrupted series of samples.

PERFORMANCE

Small cyclone

Some typical experiments only are described here, but it is to be understood that the conclusions asserted are supported by a multiplicity of experiments of each kind.

Expt. (*a*)

A suspension of *Bacillus globigii* spores, 9×10^9/ml., was sprayed into a chamber from a standard 3-jet Collison atomizer. Samples of the aerosol were taken by means of Porton impingers (May & Harper, 1957) alternately directly from the test chamber and after passing through the cyclone into a subsidiary chamber. After each sampling period the cyclone, drawing atmospheric air at 75 l./min., was scrubbed by the injection of saline; three successive samples each of 2 ml. were collected. The liquid samples were plated out in the conventional way on tryptone agar. Control samples of atmospheric air were taken into impingers; they were found to contain a negligible number of recognizable *B. globigii*.

The direct impinger counts gave for the chamber concentration 244 spores/l. (391 colonies actually counted). In the air passing the cyclone, the count was 48·5/l. (80 colonies counted). This gives an apparent gross efficiency of 80 %.

Each cyclone sampling period represented 505 l. of chamber aerosol, equivalent to 123,100 spores. The washings contained on average:

1st washings 101,700 (305 counted)
2nd washings 6,900 (104 counted)
3rd washings 3,100 (460 counted)
Ratio 1st/2nd 15:1

—total 111,700, implying an apparent gross efficiency of 91 %. The difference from the preceding figure is not significant.

This result illustrates a phenomenon found to be general: the washing of organisms from the cyclone wall is far from instantaneous, and the concentration in successive washings does not decrease in geometrical progression. The latter fact means that deposited organisms are not homogeneous in respect of their susceptibility to removal; this is not surprising, since particles of different sizes (and containing different numbers of organisms) are deposited at different levels in the cyclone. The lag in delivery to the sample receiver is due in part to the accumulation of a rotating hollow plug of liquid in the lowest part of the cyclone.

Expt. (*b*)

The numbers of organisms entering the cyclone in experiment (*a*) were much larger than is usual in field work, and the failure of the scrubbing process to follow an exponential law suggested that single organisms in small numbers might be

retained tenaciously. Under the conditions used in the preliminary experiments there was some evidence for retention.

A suspension of *B. globigii* spores, 3×10^9/ml., was sprayed from a Collison atomizer into the test chamber. At this concentration it is known that most of the aerosol particles contain one organism or none. The chamber concentration as estimated from impinger samples was 22·7 spores/l. In the air passing through the cyclone the concentration was 5·4 spores/l.—an apparent efficiency of 76 %. After a sampling period of 1 min., the cyclone was scrubbed with two portions of 2 ml. of saline while drawing clean air. The numbers recovered were

1st washings 900
2nd washings 220
Ratio 4·1 : 1

Seventy litres of aerosol passed into the cyclone during sampling, corresponding to 1590 spores; the 1120 recovered were thus 70 % of the total.

Replicate experiments of this kind gave figures of 65–70 % as the apparent collection efficiency for single spores.

Expt. (*c*)

In the previous experiments, scrubbing was carried out as a separate operation for analytical purposes. In practice, sampling and scrubbing go on simultaneously. It might be expected that particles impacted on a dry wall would be removed with more difficulty than if impacted on a surface already wetted with a film of liquid; and this was found to be true. (Experience with large cyclones is that the droplets of scrubbing liquid collect few particles while they are airborne; the spray is effective in removing particles only at and after their impaction on the walls (Stairmand, 1956). Further, the inclusion of a detergent in the scrubbing fluid greatly increased the rate of transfer of impacted organisms to the sample container; the collection efficiency as estimated from limited periods of scrubbing was increased correspondingly.

An aerosol of *B. globigii* was set up by spraying a suspension containing $8{\cdot}6 \times 10^8$ spores/ml. The cyclone sampled the aerosol for 1 min., and was then allowed to draw clean air for a further 9 min. The scrubbing liquid, a standard glycine buffer (pH 8·3), was injected throughout, and the liquid in the underflow was collected as two successive 5 min. samples. The experiment was then repeated, using as scrubbing liquid the same buffer with addition of 0·1 % (w/v) Tergitol. The numbers of spores collected were as follows (numbers actually counted in parentheses):

Scrubbing liquid	1st washings	2nd washings	Ratio
Control	18900 (189)	945 (183)	20 : 1
Tergitol	19800 (198)	300 (30)	66 : 1

Five other detergents, including cationic and anionic types, were tested similarly; Tergitol appeared to give most improvement, but Triton X100 and BRIJ 35 were hardly less effective. A non-ionic detergent is in any case to be preferred for its relative chemical inertness. We therefore adopted as standard a solution con-

taining sodium chloride, 0·11 M, sodium phosphate (pH 7·8), 0·02 M, together with 0·1 % w/v of BRIJ 35 (lauryl ether of polyoxyethylene). Using this solution as scrubbing liquid, the collection efficiency for single spores was greatly improved.

The following example illustrates the improvement, and at the same time indicates the desirability of keeping approximately constant the rate at which the liquid sample is collected.

An aerosol of *B. globigii* spores was set up as before and sampled for 1 min. into the cyclone, which thereafter drew clean air for a further 14 min. Scrubbing with the detergent saline was carried on throughout, and the liquid collected as three successive 5 min. samples. The rate of collection in the third sample was approximately twice that in the second. The result was (numbers actually counted in parentheses):

	Sample volume, ml.	Spores/ml.	Total spores
1st washings	3·3	12,910	42,620 (1033)
2nd washings	2·5	518	1296 (337)
3rd washings	4·25	378	1608 (246)

Here, although the concentration in the third washings was less than in the second, the total collected was greater. The rate at which organisms are washed out depends largely on the 'dilution rate' of the liquid accumulated above the tail pipe, i.e. the ratio of the liquid flow rate to the volume of accumulated liquid.

In this same experiment an impinger sample of the chamber aerosol taken immediately before the cyclone sample showed that 55,300 spores entered the cyclone (estimated from 589 colonies actually counted); the total of 45,000 recovered thus implies a collection efficiency of 82 %. The mean efficiency in five such experiments was 85 ± 12 %.

Our colleague G. J. Harper (unpublished) has made an extended comparison of the smaller cyclone with a number of accepted instruments of known performance. He finds its efficiency to be closely similar to that of the Litton Large Volume Sampler (Messrs. Litton Industries, Minneapolis, Minnesota, U.S.A.) and to be about 92 % that of the cascade inpactor (May, 1945), an instrument which in its modern form is sensibly perfect over most of the important range of particle diameters (say 0·5–15 μ).

Expt. (d)

Vegetative, and especially Gram-negative, bacteria are damaged by the cyclone as by some other sampling devices (May & Harper, 1957). The loss of viability actually incurred depends on the stresses the organisms have already suffered: the materials associated with them in the particles, the time for which they have been airborne, the ambient temperature and humidity, and the presence in the air of toxic substances. Because of the great potential variability, no one experiment can be considered typical. However, we found losses of 20–40 % in the viability of *Escherichia coli* aerosols sampled from a test chamber after holding times of 5–70 min., using detergent-saline scrubbing fluid.

The loss of viability can often be mitigated by suitable additions (e.g. of carbohydrates) to the sampling fluid—simple saline solutions appear to be the least

advantageous. But the best choice of a medium appropriate to the species and experimental conditions usually calls for special investigations (Cox, 1966*a*, *b*; 1967).

Another colleague, E. J. Morris (unpublished), has used the cyclone to sample aerosols containing poliomyelitis, encephalomyocarditis and Semliki Forest viruses. He finds losses of infectivity ranging from 0 to 60 %, depending on species and holding time.

Large cyclone

Because of its large sampling rate (up to 400 l./min.) we had no ready means of carrying out formal tests of collection efficiency in the larger cyclone. Since however it is designed on the same principles as the smaller, its performance would be expected to be closely similar. It was in fact found to be so by repeated comparison in the field with instruments of known performance. An example of some independent interest is described in the next section.

Estimates of the lag were obtained by an artificial procedure; for example, the cyclone was set up in the open air and run at 338 l./min. For 10 min. a saline suspension of *B. globigii* spores (about 10^3/ml.) was pumped in. Then the scrubbing fluid was changed to the standard sterile detergent-saline, and after 10 sec. the issuing liquid was collected in five successive 1 min. samples. The pump was adjusted so that the sample volumes were near 1 ml. The numbers found in the samples were

1st washing	420	(143)
2nd washing	73	(25)
3rd washing	18	(6)
4th washing	12	(4)
5th washing	0	

Ratio 1st/2nd: 5·7

Afterwards the upper and lower parts of the cyclone were separately washed with 10 ml. of saline; no *B. globigii* was found in 0·85 ml. of either washing. This last result was repeatedly obtained; it indicates that spuriously high counts due to the detachment of accumulated clumps or flakes will be rare.

A FIELD SAMPLING EXPERIMENT

A sampling station was set up on chalk downland to leeward of a ploughed area (Fig. 2). Apart from the scrub vegetation, there were no concentrated sources of biological contamination for several kilometres upwind. Three kinds of sampler were worked during the period of the experiment: the two cyclones, drawing 75 and 338 l./min., and a slit sampler drawing 28 l./min. (The slit sampler was a modification, due to K. R. May and W. C. Wright, of the original design (Bourdillon, Lidwell & Thomas, 1941); the sampler plate, charged with a tryptone agar medium, was rotated stepwise about a horizontal axis, the entrance slit being presented directly to windward.) The cyclones were fed with the buffered detergent saline solution at about 1 ml./min. and samples were taken every 5 min. for 65 min. Afterwards the samples were each made up to 5 ml. and measured volumes were plated out on tryptone agar. The plates were incubated for 36 hr. at 35°C. For the

present limited purpose all visible colonies were counted without distinction of kind; a large proportion were obviously moulds.

The meteorological conditions (2 February 1968, 10.35–11.40 a.m. G.M.T.) were:

Mean wind: 220°, 5 kt. at 2m. above ground level; 8 kt. at 10 m.

Temperature: 4·5°C.

Relative humidity: 96 %.

Sky fully overcast; very thin spasmodic rain.

During the period of sampling, a dung-spreader distributed a load of farmyard manure over the ploughed area at a distance of 600 m. upwind (Fig. 3), returned to a store 800 m. distant to be reloaded, and distributed its second load similarly.

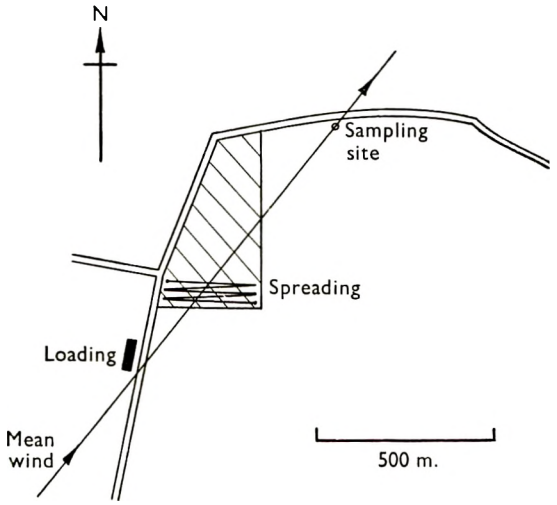

Fig. 2. Layout of field sampling experiment. Shaded area ploughed; heavy bars indicate passage of dung-spreader.

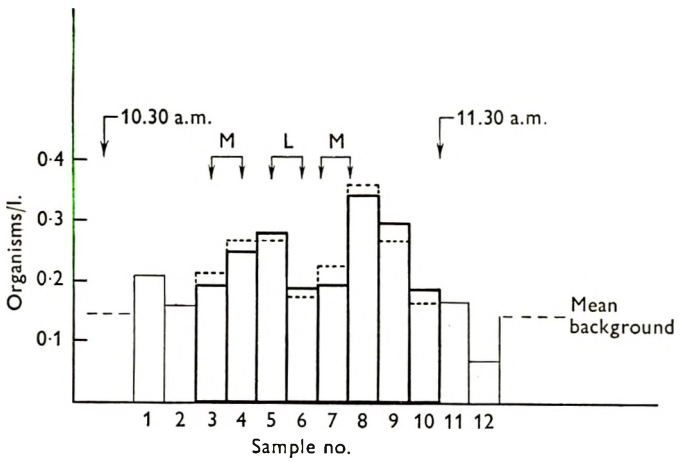

Fig. 3. Field sampling experiment. Histogram of concentrations (large cyclone); dotted ordinates corrected (Equation 3). Samples 3–10 (heavier lines) may have contained organisms derived from manuring. Activity of dung spreader indicated by yoked arrows: M, manuring; L, loading.

The timing of these operations is indicated in Fig. 3. The crude estimates of the concentration of viable particles and organisms are given in Table 1. The estimates of concentration of organisms should be regarded as nominal; apart from random sampling error, they are likely to be less than the true values because not all composite particles will be dispersed into their constituent organisms by the

Table 1. *Concentration of viable organisms in the open air, 600 m. downwind of a manuring operation*

(Numbers of colonies counted in parentheses.)

Time	Concentrations			Concentration attributable to manuring (organisms/l.)
	Slit sampler (particles/l.)	Small cyclone (organisms/l.)	Large cyclone (organisms/l.)	
10·50–	0·000(0)	0·204 (11)	0·184(40)	0·059
10·55–	0·021(3)	0·148 (8)	0·235(51)	0·092
11·00–	0·043(6)	0·074 (4)	0·262 (57)	0·097
11·05–	0·014(2)	0·185 (10)	0·179 (39)	0·028
11·10–	0·029(4)	0·204 (11)	0·184 (40)	0·069
11·15–	0·071(10)	0·259 (14)	0·322 (70)	0·185
11·20–	0·029(4)	0·241 (13)	0·280 (61)	0·015
11·25–	0·064(9)	0·167 (9)	0·179 (39)	0·013
Dosage	1·36 particle-min./l.	7·41 ± 2·11 organism-min./l.	9·12 ± 1·07 organism-min./l.	

samplers. From a total of eight samples taken outside the period of interest (10.50–11·30 a.m.) the background concentration (i.e. the concentration already in the air of organisms from distant sources) was estimated as 0·143 organisms/l. It will be seen that all three samplers indicate a maximum in concentration in the 8th (11.15–11.20 a.m.) sample; this suggests—though one uncontrolled example can hardly be taken to prove—that appreciable aerial contamination results from the process of manuring. The right-hand column of Table 1 gives the presumptive values of contaminant concentration attributable to manuring; these values were obtained by separately correcting the estimates obtained from the two cyclones (Appendix, equation (3)), forming weighted means, and deducting the background.

The dosages at the foot of Table 1 are simply the sums of the concentrations in the same column multiplied by the sampling interval (Appendix, Equation (4) with $g = 0$). The nominal dosages indicated by the cyclones (organism–min./l.) are about 6·5 times that from the slit sampler (particle–min./l.); the ratio is approximately the mean number of organisms per particle, say $\bar{z}$—again, the ratio is likely to be underestimated. Accordingly, the standard errors attached to the cyclone estimates are minimum values based on $\bar{z}$ and Σx, the total numbers of colonies counted. It will be seen that in this case the larger cyclone gives the higher estimate, but the difference is not significant. Over the whole period of the experiment (background samples included) the dosages were

Small cyclone: 10·37 ± 2·43

Large cyclone: 11·96 ± 1·32

—here again there was no indication of an appreciable difference in the gross efficiencies.

SUMMARY

Two cyclones for sampling dilute aerosols in the field are described. Their advantages are their robustness and reliability, the high degree of concentration achieved, and the ease with which they will provide a long uninterrupted sequence of samples.

Methods of correcting estimates of cloud concentration for transfer lag in the cyclone are derived.

The cyclones were constructed by J. Nott. We are indebted to G. J. Harper and E. J. Morris for allowing us to make use of their observations, and to Dr J. S. Paterson (Superintendent, Allington Farm) for providing the realistic aerosol at our convenience.

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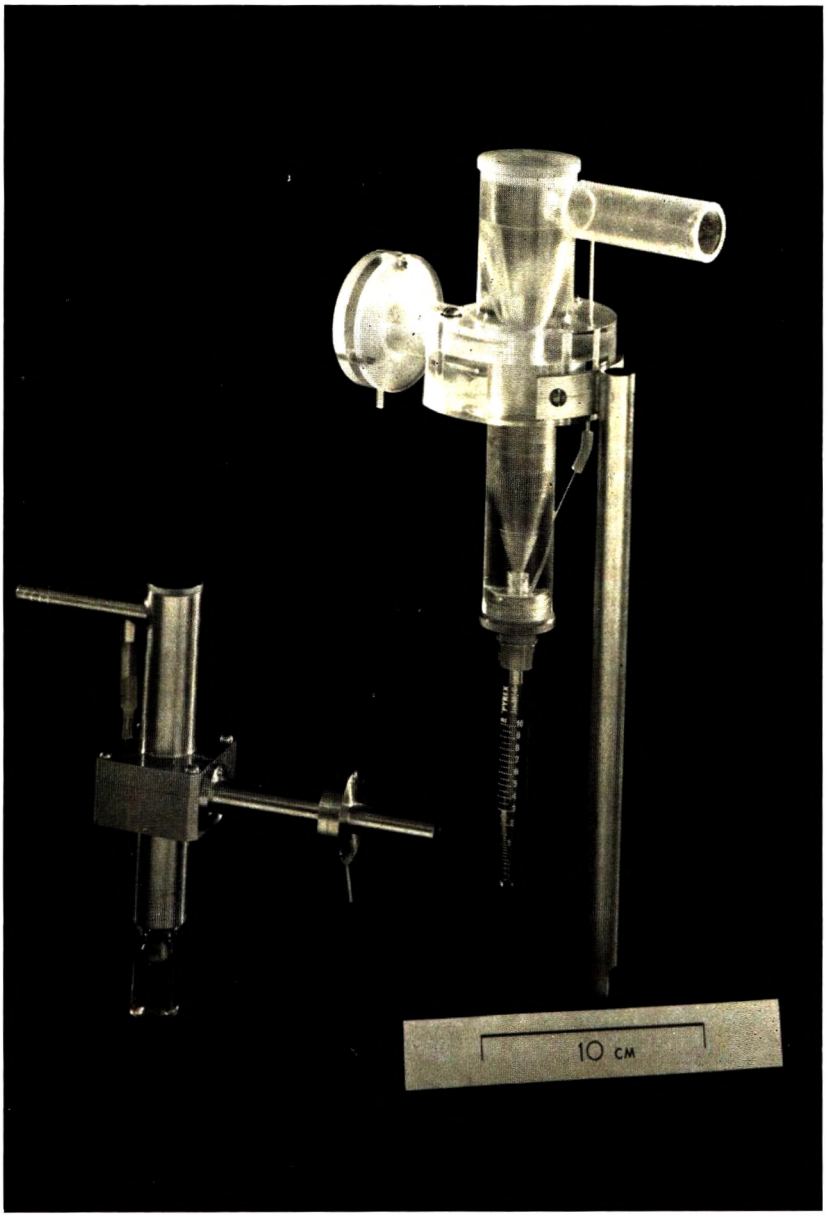
EXPLANATION OF PLATE 1

Left: Small cyclone in stainless steel with adapter for sampling from test chamber. Right: Large cyclone in Perspex with graduated sample receiver.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APPENDIX

Adjustment of data from cyclone samples

The delay in the transfer of organisms from the cyclone into the sample receiver can be characterized by a time constant (τ, say): if at any instant there are n_0 organisms within the cyclone, the number (n) of these remaining (i.e. not washed out) after time t will be given by

$$n = n_0 e^{-t/\tau}. \tag{1}$$

The lag τ can be estimated from experiments like those quoted above; if x_1 and x_2 are the numbers of organisms found in the liquid from two successive washings of equal duration Δt, and if meanwhile no new organisms enter the cyclone,

$$\tau = \frac{\Delta t}{\log (x_1/x_2)}.$$

The quantity so found will depend on the rate of scrubbing, and will appear to vary with Δt, since the removal of organisms does not follow an exponential law in fact. Only the early stages of transfer are quantitatively important, however, and a very rough value is acceptable. Thus for the first experiment quoted under (*c*) above (p. 391) the τ are 1·7 min. (control washing fluid) and 1·2 min. (detergent added). Unless τ is short compared with the sampling periods Δt, the lag is sufficient to introduce appreciable error into the estimates of a varying concentration (cf. Fig. 3). For during a sampling period some of the organisms entering the cyclone will not be washed down into the receiver; some which are washed down will have entered the cyclone during earlier periods. A correction for lag is easily made.

Suppose that a cyclone is sampling an aerosol of variable concentration $C(t)$ (organisms per unit volume) at a constant flow rate V. The rate at which organisms enter the cyclone is $VC(t)$ and the rate at which they are washed out by scrubbing is n/τ, where n is the number present (by differentiation of Eqn. (1)). Thus

$$\frac{dn}{dt} = VC(t) - \frac{n}{\tau};$$

if x is the number of organisms in the receiver,

$$\frac{dx}{dt} = \frac{n}{\tau},$$

and so

$$VC(t) = \frac{dx}{dt} + \tau \frac{d^2x}{dt^2}.$$

The number collected over a short interval, say from t to $t+\Delta t$, is now given by

$$V \int_t^{t+\Delta t} C\,(t)dt = \left[x\right]_t^{t+\Delta t} + \tau \left[\frac{dx}{dt}\right]_t^{t+\Delta t}. \tag{2}$$

Suppose now we consider three successive samples each collected over an interval Δt and containing respectively x_{r-1}, x_r, x_{r+1} organisms. Applying (2) to the middle sample, we note that

$$\int_t^{t+\Delta t} C(t)\, dt/\Delta t$$

is the mean concentration C_r in the aerosol for the period t to $t+\Delta t$, and that the first term on the right of (2) is simply x_r:

$$\Delta t V C_r = x_r + \tau \left[\frac{dx}{dt}\right]_t^{t+\Delta t}.$$

In the first of the three sampling periods, $t-\Delta t$ to t, the mean value of dx/dt is $x_{r-1}/\Delta t$; in the second, $x_r/\Delta t$. Taking their mean as an approximation for the derivative at t,

$$\left.\frac{dx}{dt}\right|_t = \frac{x_r + x_{r+1}}{2\Delta t},$$

and similarly for the derivative at $t+\Delta t$.

Hence

$$\left[\frac{dx}{dt}\right]_t^{t+\Delta t} = \frac{x_{r+1} - x_{r-1}}{2\Delta t}$$

and

$$C_r = \frac{1}{V\Delta t}\left\{x_r + \frac{\tau}{2\Delta t}(x_{r+1} - x_{r-1})\right\}.$$

Or, if we write S_r for the uncorrected estimates $(x_r/V\Delta t)$ of mean aerosol concentration in the rth sampling period,

$$C_r = S_r + \frac{\tau}{2\Delta t}(S_{r+1} - S_{r-1}). \tag{3}$$

It frequently happens that we are interested in the aerosol generated by a particular event of limited duration. In such a case the plot of concentration against time at a sampling point usually gives a bell-shaped curve, the cloud profile, but the curve may be quite erratic because of changes in wind velocity and source strength. In order to compare one profile with another, it is therefore desirable to be able to record a summary description of important features in terms of a few parameters. The first three moments of the profile form a convenient basis for such a summary. It is essential to the method that the series of samples be long enough to bracket the profile, so that its more extreme members are free from organisms derived from the source of interest, and the corresponding profile ordinates are zero.

The crude moments of the observations about a given or implied time origin are

$$m_g = \Sigma t_r^g S_r \Delta t \quad (g = 0, 1, 2), \tag{4}$$

where the t_r are the times from the origin to the centres of the sampling intervals. Corrected moments M_g are then given by (3):

$$M_g = \Sigma t_r^g C_r \Delta t = \Sigma t_r^g S_r \Delta t + \tfrac{1}{2}\tau(\Sigma t_r^g S_{r+1} - \Sigma t_r^g S_{r-1}).$$

The summations on the right can be effected by noting that

$$t_r = t_{r-1} + \Delta t = t_{r+1} - \Delta t.$$

and that, since the profile is bracketed and the extreme S_r are zero,

$$\Sigma t_{r+1}^g S_{r+1} = \Sigma t_{r-1}^g S_{r-1} = \Sigma t_r^g S r.$$

Hence

$$M_0 = m_0; \quad M_1 = m_1 - \tau m_0; \quad M_2 = m_2 - 2\tau m_1.$$

Finally we may adopt as our summary values: (i) the crude moment of zero order, m_0; this is conventionally called the 'dosage'. (ii) The reduced first moment about the origin

$$\mu_1' = M_1/M_0 = m_1/m_0 - \tau.$$

This is the time at the centroid of the profile. (iii) The reduced second moment about the centroid, namely

$$\mu_2, = M_2/M_0 - (M_1/M_0)^2,$$

is analogous to the variance of a frequency distribution and its square root is a measure of the duration of the cloud at the sampling point. Dr K. P. Norris (personal communication) has suggested that the 'passage time' should be taken as

$$2\sqrt{(3\mu_2)};$$

this is the (temporal) width of a rectangular profile having the same first three moments as the actual profile. In terms of the crude moments, and including the usual Sheppard correction for grouped data,

$$\mu_2 = \frac{m_2}{m_0} - \left(\frac{m_1}{m_0}\right)^2 - \tau^2 - \frac{(\Delta t)^2}{12}.$$

The above analysis is somewhat superficial in that it implicitly assumes the numbers and concentrations of organisms to be continuously variable quantities. A more rigorous stochastic treatment would, however, have little added practical value. It should be noted moreover that the corrected values of equation (3) are no more than improved estimates of the number of organisms actually entering the cyclone per unit volume of air passed. Regarded as estimates of the aerosol concentration itself, they are still subject to a sampling error.

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The source of bacteria in fresh cream, and the methylene blue reduction test as a guide to hygienic quality

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Attention has been focused recently on fresh cream (Colenso, Court & Henderson, 1966; Gerken, Coleman & Winner, 1968; Barrow, Miller, Johnson & Hingston, 1968; Hutchison, Barrow, Henderson & Wright, 1968) and it has been shown that a large proportion of samples examined had high bacterial counts (more than 100,000 bacteria/ml.); many contained coliform bacteria and some contained so-called faecal coliform strains. Since most of the samples had been pasteurized or manufactured from pasteurized milk, it has been assumed that the bacterial content of these creams is largely due to contamination after pasteurization. It has also been shown in these investigations that the methylene blue reduction test served as a reasonably reliable guide to the hygienic quality of fresh cream despite occasional anomalous results. The purpose of this investigation was first to examine a number of creams, identify as many as possible of the bacterial strains present and arrive at some conclusion about their source of origin. Next it was hoped to compare the results of the methylene blue test with the bacteria in the creams and study any anomalous results that might occur.

MATERIALS AND METHODS

One hundred and twenty-nine samples of fresh cream from sources in Worcestershire were brought to the laboratory by Public Health Inspectors or the County Council Milk Sampling Officer. The samples had either been heat-treated as cream or, if not, had been manufactured from heat-treated milk and were usually submitted within 2 hr. of purchase. They were examined as soon after arrival in the laboratory as possible. Viable counts were carried out by the pour-plate method using decimal dilutions of cream in $\frac{1}{4}$-strength Ringer's solution. Nutrient agar and McConkey agar incubated aerobically at 37°C. were used for the total bacterial count and coliform count. McConkey agar plates were incubated in a water-tight brass canister (Burman 1967; Barrow & Miller, 1967) immersed in a water bath at 44°C. for the so-called faecal coliform count. Plates were examined after 24 and 48 hr. incubation. Where coliform bacteria and especially *Escherichia coli* were suspected confirmatory biochemical tests (indole production test, methyl-red test and citrate utilization) were carried out. Also a loopful of undiluted cream was spread on a blood agar plate and the plate examined after 24 and 48 hr. incubation. Colonies were picked on to other blood agar plates to obtain pure cultures. Gram-staining, motility, oxidase production, catalase production and glucose fermenta-

tion/oxidation were then tested for in all strains isolated. These tests are those of the first-stage examination (Cowan & Steel, 1966). Second-stage tests (Cowan & Steel, 1966) were then used for full identification, which was possible with most of the strains examined.

For the differentiation of the coli-aerogenes type of bacteria the method recommended and described by the coli-aerogenes Sub-Committee of the Society of Applied Bacteriology (Report, 1956) was adopted. This differentiation differs from that used by Cowan & Steel (1966) in that Cowan and Steel label motile members of the genus *Klebsiella* as *Enterobacter*. A number of coli-aerogenes type bacteria remained unidentified. Some of these would have been classified as irregular types if the old nomenclature of Wilson *et al.* (1935) had been adhered to. Some difficulty was experienced in identifying saprophytes.

The methylene blue reduction test was carried out after the manner of the P.H.L.S. working party (Report, 1958), and the volumetric method used as follows. To 1 ml. of methylene blue solution prepared as for the examination of milk (The Milk (Special Designation) Regulations 1963) and 7 ml. of $\frac{1}{4}$-strength Ringer's solution in a reductase tube, cream was added to the 10 ml. mark with a wide-tipped pipette. A sterile rubber bung was then inserted, the tube was inverted once and was incubated for 17 hr. in a water bath at $20 \pm 0{\cdot}5°$C. If the mixture was still blue after this time the tube was incubated at $37 \pm 0{\cdot}5°$C. for a further 4 hr. Every half hour the tube was removed, inverted and replaced for further incubation if the blue colour had not disappeared.

RESULTS

Table 1 shows the Gram-negative bacteria isolated from the 129 samples of fresh cream, and Table 2 the Gram-positive bacteria. The remainder of the tables deal with the results of the methylene blue reduction test. Table 3 shows how the creams fell into the four grades and tables 4, 5 and 6 show the bacteria present in the samples in the various grades. Generally there was a relation between total counts of bacteria and the results of the dye-reduction test. Those creams reducing the dye during overnight incubation at $20 \pm 0{\cdot}5°$C. or this plus a short period (less than 4 hr.) at $37 + 0{\cdot}5°$C. generally had high counts; thus, of 71 creams reducing the dye overnight 53 had counts of over 100,000 bacteria/ml. (Table 6)* and of 21 creams reducing the dye in $\frac{1}{2}$–4 hr. at $37 \pm 0{\cdot}5°$C. six had counts of over 100,000 bacteria/ml. (Table 5). On the other hand, of 37 creams not reducing the dye after 4 hr. incubation at $37 \pm 0{\cdot}5°$C. only one sample had a count of over 100,000 bacteria/ml. (Table 4). There were some anomalies which will be discussed later.

* Although the upper limit of 100,000 bacteria/ml. was selected for constructing the tables, counting of bacteria was actually taken to 4 millions. Of the 53 creams in table 6 with counts of over 100,000 bacteria/ml., 45 had counts of more than 4 million bacteria/ml., and of the 6 in table 5, three had counts of more than 4 million bacteria/ml.

DISCUSSION

The origin of the bacteria isolated from the creams

Though the cream had either been pasteurized as cream or had been made from pasteurized milk many bacteria were found in the samples examined; it is therefore difficult to believe that the conditions of manufacture were hygienic. If the creams had been prepared from untreated milks the presence of most of the Gram-negative bacteria could have been explained. Most of these can usually be found in milk that has been collected in an unhygienic manner and are believed to come from the outside of the udder, the interior of the milk vessels and from dust in the milking parlour. These bacteria, however, are usually killed during pasteurization.

Table 1. *Gram-negative organisms from* 129 *samples of fresh cream*

Escherichia	
E. coli I	7
E. coli II	11
E. coli III	1
Citrobacter	
Cit. freundii I	24
Cit. freundii II	5
Klebsiella	
K. aerogenes I	22
K. aerogenes II	3
K. cloacae	12
Unidentified *Klebsiella* spp.	9
Hafnia	
Hafnia alvei	1
Alkalescens dispar group	
Alkalescens dispar	1
Unidentified coliform spp.	16
Aeromonas	
A. formicans	11
A. liquefaciens	5
Unidentified	4
Neisseria	
N. catarrhalis	9
N. pharyngis	2
N. flavescens	2
Unidentified	1
Acinetobacter	
Unidentified spp.	2
Chromobacterium	
Ch. violaceum	1
Pseudomonas	
Unidentified spp.	5
Alkaligenes	
Alkaligenes facecalis	1

Table 2. *Gram-positive organisms isolated from* 129 *samples of fresh cream*

Bacillus	
B. cereus	7
B. megaterium	5
B. subtilis	4
B. licheniformis	3
B. brevis	2
B. badius	2
B. pantothenticus	1
B. pulvifaciens	1
B. firmus	1
B. macerans	1
B. pumilans	1
B. coagulans	1
Unidentified	7
Streptococcus	
Strep. mitis	7
Strep. durans	2
Strep. bovis	4
Strep. dysgalactiae	2
Strep. faecalis	1
Unidentified streptococci	1
Aerococcus	
A. viridans	3
Corynebacterium	
Corynebacterium spp.	1
Micrococcus	
Various spp.	12
Staphylococcus	
Various spp. (non coagulase producing)	20
Staph. aureus	1

Gram-negative bacilli

When bacilli of this description, e.g. coliforms or those of the genera *Aeromonas*, *Acinetobacter*, *Chromobacter* and *Pseudomonas*, are found in cream manufactured from pasteurized milk or cream that has itself been pasteurized, the conclusion

Table 3. *Decolorization of methylene blue by* 129 *samples of fresh cream*

Time taken to decolorize methylene blue (hr.)	Number of samples examined	Grade	Number of samples with: Coliform organisms in 0·1 ml.	Number of samples with: *E. coli* in 0·1 ml.	Number of samples with plate count of (thousands per ml.): 0–1	> 1–100	> 100
0	71 (55)	IV	50 (39)	7 (5)	3 (2)	15 (12)	53 (41)
½–2	13 (10)	III	9 (7)	1	1	7 (5)	5 (4)
2½–4	8 (6)	II	2	0	4 (3)	3 (2)	1
> 4	37 (29)	I	4 (3)	0	31 (24)	5 (4)	1
Totals	129		65 (50)	8 (6)	39 (30)	30 (23)	60 (47)

Figures in parentheses are percentages of the total samples.

Table 4. *Bacteria in* 37 *fresh creams*

(These creams did not decolorize methylene blue in 4 h. at 37° C. after overnight incubation at 20 ± 0·5° C., i.e. 'passed' the test and were classed as Grade I creams.)

Bacterial count 0–1000 bacteria/ml. No. of samples = 31	Bacterial count 1001–100,000 bacteria/ml. No. of samples = 5	Bacterial count > 100,000 bacteria/ml. No. of samples = 1
B. brevis	*B. subtilis*	*B. badius*
B. megaterium	*B. licheniformis*	*Micrococcus citreus*
B. pumilis	*B. megaterium*	*Klebsiella* spp.
B. subtilis (2)	*Staph. epidermidis* (3)	
Bacillus spp. (5)	*Micrococcus* spp.	
Staph. epidermidis (2)	*Strep. bovis*	
Strep. durans	*Strep. mitis*	
Strep. bovis	*N. pharyngis*	
Aerococcus viridans	*Pseudomonas* spp.	
Mould	Coliform spp.	
M. catarrhalis	*Cit. freundii I*	
Aeromonas formicans	*Aeromonas* spp.	
Aeromonas liquifaciens		
E. coli II		
Cit. freundii I		
K. aerogenes		
Eighteen of the 31 samples did not yield growth of any bacteria.		

must be that they have been introduced after pasteurization is complete, e.g. during the handling that accompanies 'ageing', i.e. that part of the processing of cream when it is stored for at least 24 hr. at or below 40° F. to increase the viscosity, or during the filling of the cartons or bottles, and must have been derived from the lids of churns, the containers, cartons, bottles, table tops, cloths or the hands or persons of the workers.

The presence of *E. coli I* deserves special consideration. The habitat of this bacterium is the gut of humans or animals and while it is true that an accumulation of subsequent generations of *E. coli I* can be found in pipes, machinery and in or

Table 5. *Bacteria in* 21 *samples of fresh cream*

(The samples decolorized methylene blue in ½–4 hr. at 37°C. after overnight incubation at 20 ± 0·5°C., i.e. were in the intermediate position between 'failing' and 'passing' the test, and were classed as Grades II and III or 'fairly satisfactory'.)

Time taken to decolorize methylene blue (hr.)	Bacterial count 0–1000 bacteria/ml.	Bacterial count 1001–100,000 bacteria/ml.	Bacterial count > 100,000 bacteria/ml.
		Grade III	
	No. of samples = 1	No. of samples = 7	No. of samples = 5
½–2	Coliform spp.	*B. cereus* (2) *B. licheniformis* *Staph. epidermidis* (3) *E. coli I* Coliform spp. (4) *K. aerogenes I* *K. cloacae* *Aeromonas formicans* (2) *Klebsiella* spp. *Acinetobacter*	*B. megaterium* *B. pulvifaciens* *B. pantothenticus* *Staph. citreus* *Micrococcus* spp. *Strep. faecalis* *Strep. bovis* (2) *Strep. dysgalactiae* *N. catarrhalis* *E. coli II* *Cit. freundii I* *K. cloacae* *Aeromonas* spp.
		Grade II	
	No. of samples = 4	No. of samples = 3	No. of samples = 1
2½–4	*B. megaterium* *Cit. freundii I* *Klebsiella* spp. *Aeromonas* spp.	*Staph. epidermidis* *Micrococcus* spp. Coliform spp. (2) *Pseudomonas* spp. *K. ozaenae* *Aeromonas formicans*	*B. licheniformis* *Alkaligenes faecalis*

on utensils so that such contamination can hardly be said to be due to direct excretal contamination, there is no doubt at all that the presence of this bacterium in a dairy or cream manufacturing plant must have been due to excretal contamination in the first instance. Unfortunately in a number of dairies, especially those in rural areas, there is much manual filling of containers. After 'ageing', the cream is carried in vessels to a table where it is poured from a jug into cartons or bottles which are capped by hand. In some dairies those who perform these tasks are unskilled and untrained; their knowledge and understanding of hygiene is poor. Occasionally the filling room adjoins the yard; farm workers, farm vehicles and even animals pass close by; where this is so it is possible, indeed likely, that contamination with *E. coli I* is of direct excretal origin.

Gram-negative cocci

These consist of *Neisseria catarrhalis*, *N. pharyngis*, *N. flavescens* and one unidentified *Neisseria*. Usually one would label these bacteria as part of the flora

Table 6. *Bacteria in 71 samples of fresh cream*

(These samples decolorized methylene blue after overnight incubation at 20 ± 0·5°C., i.e. 'failed' the test and were therefore classified as Grade IV creams.)

Bacterial count 0–1000 bacterial/ml.	Bacterial count 1001–100,000 bacteria/ml.	Bacterial count >100,000 bacteria/ml.	
No. of samples = 3	No. of samples = 15	No. of samples = 53	
B. badius	*B. cereus*	*B. coagulans*	*Cit. freundii I* (13)
Micrococcus	*B. pumilis*	*B. megaterium*	*Cit. freundii II* (7)
Cit. freundii I	*B. macerans*	*B. subtilis*	*E. coli III*
K. cloacae	*Strep. bovis*	*B. cereus* (4)	Coliform spp. (12)
	A. viridans	*B. brevis*	*K. aerogenes I* (12)
	S. epidermidis (2)	*Bacillus* spp. (3)	*K. aerogenes II* (3)
	Micrococcus spp.	*S. epidermidis* (18)	*Hafnia*
	N. catarrhalis	*S. aureus*	*K. cloacae* (7)
	Cit. freundii I	*Micrococcus* spp. (11)	*K. ozaenae*
	K. aerogenes I	*Strep. mitis*	*K. pneumoniae* (2)
	K. aerogenes II	*Strep. durans*	*Klebsiella* spp.
	E. coli II	*Strep. dysgalactiae*	*Alkalescens dispar*
	K. cloacae (3)	*Strep.* species	*Pseudomonas* spp.
	Citrobacter spp. (4)	*A. viridans*	*A. formicans* (5)
	Coliform spp. (2)	*N. catarrhalis* (5)	*A. liquifaciens* (4)
	A. formicans	*N. pharyngis*	*Aeromonas* spp.
	Klebsiella spp. (unidentified)	*N. flavescens*	
		Neisseria spp. (2)	
		Corynebacterium	
		Acinetobacter	
		Ch. violaceum	
		E. coli I (7)	
		E. coli II (7)	

of the naso-pharyngeal passages. They have also been isolated rarely from the conjunctiva of animals. According to Wilson & Miles (1964) 'The habitat of those Gram-negative cocci that have been adequately described, with the exception of *N. gonorrhoeae* and *N. meningitidis*, is almost exclusively the nasopharynx of healthy and diseased persons and animals'. According to Breed, Murray & Smith (1957) 'All known species are parasitic'. While one would hesitate to use *Neisseria* as an absolute indicator of contamination from human or animal respiratory sources it is difficult to avoid the inference that these bacteria were present in the creams as a result of contamination by saliva and droplets from those engaged in manual filling of the containers in the course of talking, coughing and throat clearing.

Cream of course is not the only food that is liable to contamination during preparation and packing but it is a highly nutrient fluid for bacteria, often prepared and packaged under poor conditions and consumed unheated.

Gram-positive bacteria

These included a large number of the bacillus species (aerobic spore-bearers). These, of course, might have been present in the milk and survived pasteurization. They might also have been derived from the dust and air of the dairy and therefore while harmless in themselves give some indication of the degree of cleanliness of the premises.

Anomalous results

Among the 129 creams subjected to the methylene blue test were seven (5·4 %) that gave anomalous results. Three creams that did not yield a growth of bacteria at 37 ± 0·5°C. decolorized methylene blue in 2, 3 and 3½ hr. at 37 ± 0·5°C. respectively. In addition to these, three creams 'failed' the test by decolorizing methylene blue after overnight incubation at 20 ± 0·5°C. but the bacterial count in each was low. The type of organism isolated did not suggest any reason for failure. In one cream it was *Klebsiella cloacae* (300 bacteria/ml.), in another the bacteria isolated were *Bacillus badius* and *Aerococcus viridans* (700 bacteria/ml.) and in the third *Citrobacter freundii I* and *Micrococcus* spp. (100 bacteria/ml.). The type of bacteria present and the low counts would usually entitle these samples to be considered as satisfactory, yet they 'failed' the test. The seventh sample to yield an anomalous result did not decolorize methylene blue in 4 hr. at 37°C. yet *B. badius* and *Micrococcus citreus* were present in a count of more than 4 million bacteria/ml.

It was disappointing to find that the methylene blue test did not invariably pick out those creams which contained bacteria suspected of being of human or of animal origin. Thus five creams 'passed' the test although they contained bacterial species such as *Streptococcus bovis*, *Strep. durans*, *E. coli II*, *Cit. freundii I* and *K. aerogenes I*, all strains of possible intestinal origin. One cream graded as 'fairly satisfactory' yielded *Cit. freundii I*.

Value of the methylene blue test

There were seven (5·4 %) anomalous results to the methylene blue reduction test. This is a fairly high percentage and alone could possibly exclude the use of the dye test as a statutory test for fresh cream. The test also failed to identify creams that contained bacteria of possible human or animal origin. Nevertheless it served very well as a guide to the numbers of bacteria in cream and it follows therefore that it could serve as a guide to its keeping quality. It seems that it could be turned to good practical use in this respect especially if samples were examined regularly. A series of 'failures' by the test would justify examination of premises, equipment and methods to discover the sources of contamination.

SUMMARY

One hundred and twenty-nine samples of fresh cream collected in Worcestershire were examined bacteriologically. Sixty (46·5 %) creams had counts of over 100,000 bacteria/ml. The bacteria present were of many varieties, the commonest being *Bacillus* spp. (aerobic spore formers), Gram-negative bacilli, staphylococci

and micrococci. Since most of the creams had been either pasteurized as cream or manufactured from pasteurized milk it was thought that the many bacteria were present because of contamination after pasteurization due to three main causes; unsatisfactory or unhygienic premises, unsuitable equipment, manual handling during the filling and capping process.

The methylene blue test results related well with bacterial counts but there were seven (5·4 %) anomalous results. Although the methylene blue reduction test therefore could serve as a simple and reasonable guide to the hygienic quality of fresh cream, 5·4 % of anomalous results would perhaps make it unsuitable as a statutory test.

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Brucellosis in Northern Ireland. A serological survey

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INTRODUCTION

The eradication of brucellosis in cattle has been considered a necessary preliminary to the control of disease in man (Bothwell, 1960; Bothwell *et al.* 1963; Anon. 1966), for even when all milk marketed for human consumption is pasteurized, a percentage of the population living in the rural areas will consume unpasteurized milk. In Northern Ireland, this group has been estimated previously as high as 400,000 people or roughly one-fourth of the total population (Kerr & Rankin, 1963).

Britain has no brucellosis eradication programme, but in Northern Ireland a scheme for grade A herds was introduced in 1959 (Milk (Amendment No. 2) Regulations, 1959), which was first widened in scope to include the remaining dairy herds voluntarily (Brucellosis Order, 1960) and then made compulsory in 1963 (Brucellosis Control Order, 1962). By December 1967, 15,527 out of the total of 16,300 dairy herds were certified brucella-free whilst eradication was proceeding in the remainder. In addition, 23,000 herds from a total of 28,000 beef and suckling herds had also reached certified status and 3,500 were under test (Christie, Kerr & McCaughey, 1968).

Pasteurization of all milk supplies except those from Grade A herds has been enforced since 1950 (Milk Act (Northern Ireland), 1950) and brucellosis has been eradicated from these herds since 1961.

During 1967 a survey was carried out in Northern Ireland to determine the prevalence of antibodies to *Brucella abortus* in the population. It was thought that it might give some indication of the initial effect of the eradication programme for bovine brucellosis on the amount of human exposure and disease. The results of the survey are reported in this paper.

METHODS

Sera from the various groups were examined for brucella antibodies by the standard agglutination test, the anti-human globulin (A.H.G.) (Coombs) test, and the complement fixation test.

Serum agglutination test

This was carried out by the method described by Alton & Jones (1967) at serum dilutions from 1/10 to 1/5120. Any sera showing agglutinations at 1/5120 were taken to titre. The antigen used was an agglutinable suspension of *Brucella abortus* Strain 99 standardized to give 50% agglutination with a 1/500 dilution of International Standard Serum.

Anti-human globulin (A.H.G.) (Coombs) test

The method of Wilson & Merrifield (1951), as modified by Kerr, Coghlan, Payne & Robertson (1966*b*) was used. The antigen was the same as described above. Anti-human precipitating rabbit serum (Burroughs-Wellcome) was used at its optimal dilution.

Complement-fixation test

This test was performed as described by Bradstreet & Taylor (1962), by a 4 volume test (unit volume 0·1 ml.) in W.H.O. plastic plates. The short fixation method only was used with 2·0 M.H.D. of complement and the optimal dilution of antigen (heat-killed *Brucella abortus* Strain 99) as determined by a chessboard titration. In each series of tests a known control serum was included.

RESULTS

A total of 1,894 sera were tested. The results are shown in Table 1.

Blood transfusion sera

One thousand and seventy eight sera were obtained from the Northern Ireland Blood Transfusion Service. They originated from a variety of centres, including Belfast, Dungannon, Lisburn and Magherafelt. The majority of donors lived in these centres and thus, whilst all these sera were designated 'urban', a very small proportion of them may have come from rural dwellers. A very small proportion of the sera, never more than 2·7%, showed any evidence of possessing antibodies to *Br. abortus* whichever test was used. The serological titres were always low.

Farmer's lung sera

Two hundred and fifty two sera were included which had been submitted to the Mycology Diagnostic Laboratory with a possible diagnosis of Farmer's Lung. These sera were not necessarily from farmers or farm workers, but it was assumed that the individuals concerned had contact with farms or a rural setting, as only three out of 601 specimens submitted over a period came from Belfast Borough.

The proportion of sera which gave reactions to the serum agglutination test was similar to that of the urban group, but with the A.H.G. and complement-fixation tests this rural group showed a higher proportion of reactions than the urban group.

Veterinary surgeons

There were 125 veterinary surgeons in private practice in Northern Ireland in 1967, of whom 118 gave blood for examination. They were predominantly engaged

Table 1. *Serological titres of the different groups investigated*

Group	Total no. in group	Standard agglutinations titre				Anti-human globulin titre				Complement-fixation titre			
		⩾1/10	⩾1/20	⩾1/40	⩾1/80	⩾1/10	⩾1/20	1⩾/40	⩾1/80	⩾1/4	⩾1/8	⩾1/16	⩾1/32
Blood transfusion sera (urban)	1078	23 (2·1)	2 (0·2)	0	0	29 (2·7)	4 (0·4)	1 (0·1)	0	2 (0·2)	2 (0·2)	0	0
Farmer's lung sera (rural)	252	6 (2·4)	2 (0·8)	1 (0·4)	1 (0·4)	19 (7·5)	9 (3·6)	3 (1·2)	1 (0·4)	6 (2·4)	5 (2·0)	5 (2·0)	4 (1·6)
Vets in private practice	118	34 (28·8)	20 (16·9)	8 (6·8)	3 (2·5)	110 (93·2)	107 (90·7)	92 (78·0)	76 (64·4)	84 (71·2)	78 (66·1)	69 (58·5)	55 (46·6)
Vets in Ministry practice	78	7 (9·0)	5 (6·4)	0	0	50 (64·1)	44 (56·4)	26 (33·3)	17 (21·8)	29 (37·2)	25 (32·1)	18 (23·1)	12 (15·4)
Non-qualified Ministry helpers	26	4 (15·4)	4 (15·4)	2 (7·7)	0	8 (30·8)	6 (23·1)	5 (19·2)	2 (7·7)	6 (23·1)	5 (19·2)	5 (19·2)	5 (19·2)
Artificial insemination workers	7	0	0	0	0	0	0	0	0	0	0	0	0
Abattoir workers	243	22 (9·1)	13 (5·3)	8 (3·3)	6 (2·5)	35 (14·4)	30 (12·3)	8 (3·3)	8 (3·3)	20 (8·2)	7 (2·9)	0	0
Dairy farmers or farm workers	92	20 (21·7)	14 (15·2)	4 (4·3)	2 (2·2)	40 (43·5)	32 (34·8)	25 (27·2)	14 (15·2)	16 (17·4)	9 (9·8)	6 (6·5)	3 (3·3)
Total	1894	116 (6·1)	60 (3·2)	23 (1·2)	12 (0·6)	291 (15·4)	232 (12·2)	160 (8·4)	118 (6·2)	163 (8·6)	131 (6·9)	103 (5·4)	79 (4·2)

Figures in parentheses are percentages of totals for each group.
⩾ 1/10 includes figures for ⩾ 1/20 etc.

Table 2. *Serological titres related to occupational exposure to cattle*

Group	Total no. in group	Standard agglutination titre				Anti-human globulin titre				Complement-fixation titre			
		⩾ 1/10	⩾ 1/20	⩾1/40	⩾ 1/80	⩾1/10	⩾ 1/20	⩾1/ 40	⩾ 1/80	⩾ 1/4	⩾ 1/8	⩾ 1/16	⩾ 1/32
No definite exposure to cattle	1330	29 (2·2)	4 (0·3)	1 (0·1)	1 (0·1)	48 (3·6)	13 (1·0)	4 (0·3)	1 (0·1)	8 (0·6)	7 (0·5)	5 (0·4)	4 (0·3)
Definite occupational exposure to cattle	564	87 (15·4)	56 (9·9)	22 (3·9)	11 (1·9)	243 (43·1)	219 (38·8)	156 (27·7)	117 (20·7)	155 (27·5)	124 (22·0)	98 (17·4)	75 (13·3)
Total	1894	116 (6·1)	60 (3·3)	23 (1·2)	12 (0·6)	291 (15·4)	232 (12·2)	160 (8·4)	118 (6·2)	163 (8·6)	131 (6·9)	103 (5·4)	79 (4·2)

Figures in parentheses are percentages of totals for each group.
⩾ 1/10 includes figures for ⩾ 1/20. etc

in 'large animal' practice and spent between 50 and 75 % of their time with cattle. The Ministry of Agriculture employed a further 108 veterinary surgeons during this period; 78 sera were obtained from this group. Of these, approximately one fourth worked in an administrative capacity and two worked in artificial insemination centres, but the majority were employed in bovine tuberculosis and brucellosis eradication programmes or carried out meat inspection duties. Owing to the different nature of their employment, these two groups were considered separately.

The proportion in each group showing reactions to the serological tests was higher than in either the urban or rural groups. Also a greater proportion of veterinary surgeons in private practice had positive tests than those in Ministry employment. All three tests showed these differences, but the A.H.G. and complement-fixation tests gave many more positive results than the standard agglutination test. Many of the sera were positive in the higher dilutions by the A.H.G. and complement-fixation tests.

Ministry of Agriculture non-veterinary assistants

The Ministry of Agriculture also employ non-veterinary field staff to assist in eradication and meat inspection. Their work is mainly clerical but they do have some practical duties. Twenty-six sera were examined from this group. Their tests showed some evidence of exposure to brucella antigen, but the number was small and detailed comparison not possible.

Artificial insemination workers

The workers from one of the Ministry of Agriculture Artificial Insemination Plants were also submitted to serological investigation. There were only seven sera and all of them were negative to all three tests. In addition, the results of the Ministry veterinary surgeons include two qualified veterinary surgeons working in artificial insemination. Their tests were entirely negative.

Abattoir workers

Abattoir and meat packing plants in Northern Ireland employed 1005 people in 15 different centres in 1967. From these 243 sera were obtained.

This group showed an incidence of reactions considerably higher than the rural and urban groups with all three tests. Except with the complement-fixation test, many of the sera were positive in the higher dilutions.

Dairy farmers or farm workers

Ninety-two sera were obtained from farmers or farm workers engaged in dairy farming in Northern Ireland; these were divided between Counties Armagh, Down, Fermanagh and Londonderry. No samples were available from Counties Antrim or Tyrone.

The serum agglutination test in this group gave a proportion of reactions similar to but rather lower than those of the veterinary surgeons in private practice. There was also a considerable number of reactions with the other tests, some of which persisted at higher dilutions.

Occupational exposure

The influence of occupation in Northern Ireland on the prevalence of serologically positive tests for brucella antibody can be seen from Table 2.

Sera of persons in Northern Ireland with no known exposure to cattle, whether living in urban or rural settings, showed a very low incidence of serological positives for *Br. abortus* when measured by the standard agglutination test. In fact, only one out of 1330 sera in this category had a titre as high as 1/80. Those working in occupations bringing them into contact with cattle showed a much greater proportion of positives. The amount of exposure and subsequent antibody production seemed to vary with occupation. Veterinary surgeons in private practice contributed most to this group, but those employed by the Ministry in field work, by abattoirs or in dairy farming were also very much at risk.

More positives were obtained with either the A.H.G. test or the complement-fixation test than with the serum agglutination test in groups which were occupationally exposed. These tests were often positive at high dilutions.

DISCUSSION

In the only previous serological survey for brucellosis in Northern Ireland, Murdock (1944), using the standard agglutination test, found 6·5 % of 2073 sera submitted by blood donors with a titre of 1/40 or greater against *Br. abortus*. He considered part of his sample to be rural. In this present study, even including 252 sera from what was thought to be a rural and probably a symptomatic group, only 0·1 % of 1330 sera had a titre as high as 1/40. This suggests that there is now a smaller proportion of persons with brucella antibodies in Northern Ireland, but the lack of standardization in the tests and in the materials used for the tests makes exact comparisons between such surveys difficult.

Murdock estimated that, if the proportion of positive results in his series was representative of the whole province, the total number expected to show specific agglutinins against *Br. abortus* would have been about 81,000. From this present study the comparable figure would be 1500, though the population has risen by approximately 250,000 to one and a half million.

Recent brucellosis studies in England on blood transfusion sera give conflicting reports. In the Isle of Wight, Brodigan, McDiarmid, Mann & Skone (1961) found about 17 % to have brucella antibodies, with 5 % showing a titre of at least 1/80. However, Bartram *et al.* (1963) in a large series from the Oxford area, found only 1·1 % positive, with 0·4 % at higher titres. Both of these surveys were carried out between 1957 and 1959 with the serum agglutination test. Bartram *et al.* (1963) suggested that different exposure to raw, potentially infected milk might account for the wide variation between the findings of these two surveys. In 1959–1960, 95 % of milk was pasteurized in the Oxford area, but only 77·3 % in the Isle of Wight. Also, Brodigan *et al.* (1961) had found positive titres in 70 % of sera from 27 persons known to be drinking raw milk infected with brucella organisms.

Murdock believed that the exposure encountered in his survey was milk-borne,

and it has long been held that ingestion of raw infected milk is the principal method of transmission of brucellosis in Great Britain (Dalrymple-Champneys, 1960; Bothwell, 1963; Parry, 1966). The brucellosis eradication in cattle, together with the amount of pasteurization of milk and the special regulations pertaining to milk retailed raw in Northern Ireland may explain why Murdock's figures differ from this present survey; but this fails to explain why, despite the differences in milk regulations and brucellosis legislation, the figures for the Oxford survey and the present Northern Ireland one are similar.

There are several factors which may contribute to this. Firstly, the surveys may not be comparable; the difficulties relating to laboratory standardization have already been mentioned. Secondly, as far as the general population is concerned, milk may be the vehicle of infection and the amount of unpasteurized milk consumed may be similar in the two areas. Thirdly, a proportion of normal individuals may possess antibodies which agglutinate *Brucella abortus* at low titres. Lastly, serological titres may persist for long periods, even up to 10 years, despite the absence of continuing exposure to infection (Dalrymple-Champneys, 1929; Bartram *et al.*, 1963; McDevitt, 1968). Thus it may be too early to assess the effects of the brucellosis eradication programme by this means.

The results of this survey suggest that brucellosis or certainly the acquisition of brucella antibodies in Northern Ireland is now mainly an occupational hazard, as in America (Spink, 1956). However, it is worth remembering that people, occupationally exposed to infected animals, with the exception of abattoir workers, are also more likely to have access to raw milk (Barrett & Rickards, 1953; Bothwell, 1963). Close proximity to cattle is not the only factor as the Ministry veterinarians and their helpers, whose duties relate mostly to sampling blood, milk or vaginal mucus, seem less exposed than veterinary surgeons in private practice or dairy farmers. The major hazard appears to be the intimate contact with material infected by *Br. abortus* that bovine 'midwifery' involves, and, although veterinary surgeons in private practice are most at risk (Huddleson & Johnson, 1930; Kerr, Coghlan, Payne & Robertson, 1966*a*), farmers and farm workers may also acquire infection in this way.

Handling infected carcasses is a real hazard, as is shown by American experience in the meat packing industry (Spink, 1956). Brucellosis eradication has increased the number of infected animals being slaughtered, and, if the farmer claims that his condemned cow is pregnant, which entitles him to increased compensation, then careful examination is required. Adequate safety precautions at this point are, therefore, necessary and justify more attention.

Various authors in previous surveys have commented on the greater sensitivity of the A.H.G. test in the detection of 'incomplete' brucella antibodies (Ferris, Stevenson & Lewis, 1953; Coetzee, 1956; Zoutendyk, 1958; Schrire, 1962). The results of this present study would confirm this. In the occupationally exposed groups, the A.H.G. and complement-fixation tests revealed many more positives than the direct agglutination test; this was not so with the groups of blood transfusion and farmer's lung sera so far as the complement-fixation test was concerned. More sensitive tests might be clinically useful, but it is worth remembering that a

positive serological test in a patient does not necessarily prove a diagnosis of active brucellosis.

Enthusiasm for these more sensitive tests may be reflected in the number of cases of brucellosis notified to the Ministry of Health and Social Services in Northern Ireland. Whereas 63 cases were reported between 1949 and 1959 (Bothwell, 1963), and 20 cases were notified from 1960–1966, 44 cases were notified in 1967 alone (T. T. Baird, pers. comm.) at a time when, despite the brucellosis eradication programme, interest was being renewed in the A. H. G. test as a diagnostic tool in human brucellosis (Kerr *et al.* 1966*b*) and facilities for carrying it out were becoming available in the province.

Although it may be too early to assess the effects of the eradication of bovine brucellosis in the human population by serological methods, this survey does provide a basis from which the future effects may be estimated.

SUMMARY

A serological survey for brucella antibodies has been carried out in Northern Ireland in an attempt to assess the effects of the brucellosis eradication programme in cattle which has been in progress since 1959. One thousand eight hundred and ninety four sera from blood donors, from farmer's lung tests, and from various groups occupationally exposed to cattle were examined by the serum agglutination test, the anti-human globulin (A.H.G.) (Coombs) test and the complement-fixation test.

The incidence of brucellosis in Northern Ireland is now less than it was 20 years ago, but is similar to that reported in a recent survey in England. It now appears that the principal method of transmission of brucellosis in the province is by occupational exposure rather than by drinking infected milk. Though it may be too early to make a serological assessment of the effects on human infections of brucellosis eradication in cattle, this survey provides a basis for future evaluation.

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Transfer areas and clean zones in operating suites

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It is generally assumed that floors in the aseptic zone of operating suites should be protected against contamination with bacteria brought in from other parts of the hospital. The use of plastic overshoes and theatre boots are convenient measures against contamination from the soles of shoes, but it is harder to devise practical methods of preventing contamination from the wheels and other surfaces of trolleys. The use of tacky mats or disinfectant dips for this purpose is of rather doubtful value (Ayliffe *et al.* 1967; Medical Research Council, 1968).

A design of operating suites has been recommended in which the aseptic ('sterile') zone, including the operating and sterilizing rooms, is approached through a clean zone, where theatre clothes are worn; the clean zone is approached through a protective zone, where the staff changes into theatre clothes, footwear, etc. (Medical Research Council, 1962). Between the protective and clean zones in some operating suites a transfer area is provided, where patients are transferred from the hospital trolleys, on which they are brought from the wards, to clean theatre trolleys; this arrangement is thought to have some value in protecting the aseptic zone against contamination from trolleys (Weeks, 1964; Barron, 1964).

The presence of such an area with clean trolleys requires a considerable addition to the floor space allocated for the operating suite; transfer to fresh trolleys involves additional handling of sick patients, and also some congestion and delay in conveying patients to the operating room. The inclusion of transfer areas in plans of new hospitals can be justified only if it is shown to reduce the hazards of infection by reducing the levels of bacterial contamination in the aseptic zone.

In the study reported here we attempted to answer three questions: (1) does the presence of a transfer area reduce the amount of contamination introduced into the clean and aseptic zones of operating suites? (2) are the clean and aseptic zones of operating suites provided with transfer areas cleaner than those without transfer areas? (3) is the presence of a clean zone associated with less contamination in the aseptic zone than that which is found in theatre suites with no clean zone? The role of the environment as a source of infection with *Clostridium welchii* is also discussed.

METHODS

Theatres

Studies were made in the operating suites of two hospitals, including one suite with a transfer area and one with neither clean zone nor transfer area. Nine other operating theatres, including suites in eight other hospitals, were also studied in less detail.

The operating suite in Hospital 1 is of recent design; it is ventilated with a plenum system and consists of well-defined protective, clean and aseptic zones. The protective zone is separated from a clean zone by a trolley transfer area and changing rooms. The patient is transferred from a hospital trolley to a theatre trolley in this area. Ward staff accompany the patient to the transfer area and theatre staff accompany the patient to the anaesthetic room and the operating theatre. Ward staff do not enter the clean or aseptic zone without changing into theatre clothing and theatre staff should not leave the clean zone in theatre clothing or footwear. Theatre trolleys are used in the clean and aseptic zones only and are cleaned weekly or on special occasions if necessary. Hospital trolleys are also cleaned weekly and never pass beyond the transfer area into the clean zone. Occasionally, a patient is taken from the theatre to the ward on a theatre trolley (if too ill to be transferred to another trolley). The trolley is then cleaned before being returned to the clean zone. If the patient's bed is required in the theatre, the bed is cleaned before being taken into the clean zone. Floors of protective and clean zones are mopped daily and more thoroughly washed and polished weekly. Theatre floors are cleaned after each operating session with a detergent and water.

Two operating suites were investigated in Hospital 2. Suite A is of recent design, plenum ventilated and with protective, clean and aseptic zones, but no transfer area. Staff entering the clean zone change to theatre footwear or put on plastic overshoes. Suite B has no mechanical ventilation system or clean zone, and personnel in outdoor clothes and shoes may walk up to the doors of the operating theatre. In both of these operating suites patients are brought into the theatre without changing trolleys and returned from the theatre to the ward on the same trolley. The floors of the suites are washed with a disinfectant at least once a day, and trolleys are washed at irregular intervals.

None of the nine other operating suites that were studied in less detail had a transfer area, and the presence of a plenum ventilation system or well-defined clean zone was variable. In all the theatres studied, bedding from the ward was removed before the patient was taken into the clean zone, or into the theatre if there was no clean zone.

Bacteriological methods

Alne disposable surface contact plates were used for sampling floors, trolley wheels and footwear. These plates are 6·25 cm. in diameter and are marked with a grid of 21 1 cm. squares; they are a modification of sampling plates described by Hall & Hartnett (1964). The plates are filled with an agar medium (approximately 18 ml.) to provide a surface raised slightly above the rim of the plate. After drying, the plate is pressed firmly on the surface to be sampled. Nutrient agar, containing

phenolphthalein diphosphate (P.P.D. medium, Barber & Kuper, 1951), was used for total counts and counts of presumptive *Staphylococcus aureus*, and Neomycin-Nagler agar (N.N.A. medium, Lowbury & Lilly, 1955), was used for counts of *Cl. welchii*. Swabs moistened with peptone water were used for sampling trolley wheels and the framework of trolleys; they were rubbed over half of the surface of a P.P.D. plate, and this primary inoculum was spread with a loop over the other half of the plate. Counts of colonies on all plates were made after 18 hr. incubation at 37°C. Five colonies or 10% of the colonies of presumptive *Staph. aureus* (whichever was the higher) were examined for coagulase production by the slide method and a selection of colonies from N.N.A. plates was examined for inhibition of lecithinase by *Cl. welchii* antiserum. All presumptive *Staph. aureus* and *Cl. welchii* were confirmed in this sample of strains.

Details of sampling

Trolleys

Wheels. Two samples were taken from the outer surface of the tyre of each wheel with a contact plate containing N.N.A. medium. A swab, moistened with Ringer's solution, was rubbed over a further 6·0 cm. of the tyre of each wheel. Twenty-four wheels from six theatre trolleys and 24 wheels from six hospital trolleys were examined in Hospital 1. Samples were taken 6 days after the trolleys were cleaned. Twenty trolley wheels from five trolleys in Hospital 2 were similary sampled. Trolleys used in clean and aseptic zones only are referred to as 'Theatre trolleys', trolleys used in the hospital but not in the theatres are referred to as 'Hospital trolleys', and trolleys used both in the hospital and in the theatres are referred to as 'Hospital and Theatre trolleys'.

Handles, bars and tops. A moistened swab was run along the whole length of the trolley handle on the upper and lower surface. Moistened swabs were also rubbed over the surface of approximately 6 cm. of an upper bar near the top of the trolley and of a lower bar. Samples were also taken from the top of the trolley with contact plates.

Floors

Samples were taken from hospital corridors adjacent to the theatre suites and from protective zones and theatres in the three theatre suites in Hospitals 1 and 2 and also from the clean zones in Hospital 1 and in theatre A of Hospital 2. The transfer area in Hospital 1 was also sampled. Two visits for sampling were made to each theatre suite. From ten to 28 samples were taken in each area.

Floor and air samples were also taken in 11 theatres during single operating sessions. In each theatre 250–750 ft^3 of air was sampled with a slit-sampler on an N.N.A. plate, and in five of the theatres ten contact plates containing N.N.A. were taken at random sites from the floors.

Theatre footwear and outdoor shoes

Theatre shoes or boots and outdoor shoes from three hospitals were sampled. The outdoor shoes were sampled in theatre changing rooms. A sample was taken from

the heel and sole of each pair of boots or shoes with a contact plate. The heel of one shoe and the sole of the other shoe in each pair was sampled with a plate containing N.N.A. and the opposing heel and sole with a plate containing P.P.D. agar. Theatre footwear was washed weekly, or more often if contaminated, in the theatres studied.

RESULTS

Trolleys

Bacterial counts from trolley wheels are shown in Table 1. The mean counts of *Cl. welchii* from theatre trolley wheels were significantly lower than those from the wheels of hospital trolleys in the same hospital ($t = 26{\cdot}63$, $P < 0{\cdot}001$); there was a similar difference between the counts of *Cl. welchii* on these theatre trolley wheels and hospital trolley wheels in the other hospital (2).

Table 1. *Bacterial contamination of trolley wheels*

Hospital		Number of samples	Mean total organisms per plate	Mean *Staph. aureus* per plate	% of plates showing *Staph. aureus*	Mean *Cl. welchii* per plate
1	Theatre trolleys	24	550	18·6	12·5	3·13 ± 0·47
1	Hospital trolleys	24	834·5	18·6	41·7	67·90 ± 7·68
2	Hospital and theatre trolleys	20	287·6	6·0	35	45·10 ± 3·90

Table 2. *Bacterial contamination of trolleys*

Hospital		Site of sampling	Number of samples	Mean total organisms per plate	Mean *Staph. aureus* per plate	Mean *Cl. welchii* per plate
1	Theatre trolleys	Top	12	48·2	0·42	0·75
		Bars	12	89·3	2	—
		Handles	6	12·3	0	—
1	Hospital trolleys	Top	12	36·8	5·6	0·92
		Bars	12	88·7	0·17	—
		Handles	6	12·6	0	—
2	Hospital and theatre trolleys	Top	10	35·2	0·33	0·5
		Bars	10	9	0·1	—
		Handles	5	11·4	0	—

Mean counts of total organisms were lower from wheels of theatre trolleys than from the hospital trolleys in the same hospital, but both were higher than from the wheels of the hospital-and-theatre trolleys of Hospital 2. The mean count of *Staph. aureus* was high from the theatre trolley wheels; this was due mainly to one heavily contaminated wheel, and in the theatre trolleys the percentage of wheels contaminated with *Staph. aureus* was lower than it was in the hospital trolleys or in the trolleys from the other hospital. Table 2 shows the bacterial contamination of the trolley top and framework. Mean total counts were low from all areas,

particularly the bars of trolleys from Hospital 2. Mean counts of *Staph. aureus* were also low; the higher mean count from the tops of hospital trolleys in Hospital 1 was due mainly to one plate showing 50 colonies.

Floors

Table 3 shows the mean bacterial counts from the operating suite of Hospital 1, and Table 4 from operating suite A of Hospital 2.

In both suites a significantly lower mean count of *Cl. welchii* was found in the clean zone than in the hospital corridor (Hospital 1: $t = 10{\cdot}04$, $P < 0{\cdot}001$;

Table 3. *Bacterial counts of floors*

(Hospital 1 with transfer area and clean zone.)

	Total organisms and *Staph. aureus*			*Cl. welchii*	
Site of sampling	Number of plates	Mean total organisms per 100 cm².	Mean *Staph. aureus* per 100 cm².	Number of plates	Mean per 100 cm².
Hospital corridor	15	483·3	1	15	36·67 ± 13·37
Protective zone	20	469	8·33	20	41·17 ± 10·57
Transfer area	15	379·3	1·67	15	65·77 ± 15·13
Clean zone	25	295·7	1·33	25	2·80 ± 2·07
Theatre	20	111	0	20	0·83

Table 4. *Bacterial counts of floors*

(Hospital No. 2, Theatre A, no transfer area.)

	Total organisms and *Staph. aureus*			*Cl. welchii*	
Site of sampling	Number of plates	Mean total organisms per 100 cm².	Mean *Staph. aureus* per 100 cm².	Number of plates	Mean per 100 cm².
Hospital corridor	15	1052·3	8·7	20	50·50 ± 5·83
Protective zone	18	336·0	2·0	20	11·50 ± 1·87
Clean zone	28	206·0	1·7	20	1·33 ± 1·40
Theatre	18	283·3	1·0	20	0·5

Hospital 2, theatre A: $t = 10{\cdot}34$, $P < 0{\cdot}001$). The protective zone in theatre A of Hospital 2 shows much lower counts of *Cl. welchii* than those found in the protective zone of Hospital 1; the transfer area in Hospital 1 shows higher counts of these organisms than were found in the hospital corridor or protective zone. However, the low counts from clean zones and theatres in both suites suggest that transferring patients to clean trolleys has little effect on floor contamination in these zones. Mean total counts and counts of *Staph. aureus* also showed reductions

between contaminated, clean and aseptic areas, but no marked difference between the two theatre suites.

Table 5 shows the results from theatre B in Hospital 2. This theatre has no transfer area or defined clean zone. Mean counts of *Cl. welchii* per plate show a reduction between the hospital corridor and protective zone similar to that found in theatre A, but there was a higher mean count of *Cl. welchii* on the floor of the aseptic zone (theatre) in this suite (20·5) than in the two other suites (0·83 and 0·5). This was due mainly to two plates with counts of 71 and 24 colonies respectively. The plates with high counts were taken from near the theatre doorway. The mean count from the theatre floor was significantly lower than the count from the corridor ($t = 3{\cdot}70$, $P < 0{\cdot}001$).

Table 6 shows counts of *Cl. welchii* in the air from studies of eleven theatres, including two of those already described, and from the floors of five theatres. The counts of *Cl. welchii* in the air and on the floor were generally low, although the counts in the air were higher in theatres without plenum ventilation.

Table 5. *Bacterial counts of floors*

(Hospital No. 2, Theatre B, no transfer area or clean zone.)

	Total organisms and *Staph. aureus*			*Cl. welchii*	
Site of sampling	Number of plates	Mean total organisms per 100 cm².	Mean *Staph. aureus* per 100 cm.²	Number of plates	Mean per 100 cm².
Hospital corridor and doorway	15	560	2·33	15	102 ± 19·4
Protective zone	15	346·67	0·33	15	15·1 ± 3·6
Theatre	10	286·67	0·33	20	20·5 ± 12·33

Table 6. Cl. welchii *in the air (slit-sampling) and on the floor of operating theatres*

Hospital	Plenum ventilation	Clean zone	Transfer area	*Cl. welchii* per 100 ft³ of air	Mean *Cl. welchii* per 100 cm.² (10 plates)
1	Yes	Yes	Yes	0·7	2·67
2A	Yes	Yes	No	0·4	2·33
C	No	No	No	3·6	2·33
3	Yes	Yes	No	0	0·67
4	Yes	No	No	0·3	1·67
5	No	No	No	1·3	—
6	No	No	No	3·4	—
7	Yes	Yes	No	0·5	—
8	Yes	Yes	No	0·8	—
9	No	No	No	1·4	—
10	No	Yes	No	0	—

— Not tested

Footwear

Bacterial counts from theatre shoes or boots and outdoor shoes from the theatre suites of three hospitals are shown in Table 7. Mean total counts and counts of *Staph. aureus* were much lower in samples taken from theatre footwear than from outdoor shoes. The mean count of *Cl. welchii* was significantly lower from theatre shoes than from outdoor shoes ($t = 28{\cdot}9$, $P < 0{\cdot}001$).

Table 7. *Bacterial contamination of theatre footwear and outdoor shoes from three hospitals*

	Total organisms and *Staph. aureus*			*Cl. welchii*	
Type of footwear	Number of samples	Mean total organisms per plate	Mean *Staph. aureus* per plate	Number of samples	Mean per plate
Theatre	40	360	1·1	44	10·45 ± 0·98
Outdoor	36	> 1,000	10·6	40	74·05 ± 10·52

DISCUSSION

From earlier studies on the bacteriology of air in a hospital (Lowbury & Lilly, 1958) it was concluded that *Cl. welchii*, unlike *Staph. aureus*, is introduced into buildings from the exterior; there is little evidence that it is dispersed by human carriers inside the building. These conclusions are also supported by results described here. *Cl. welchii* therefore appears to provide a good index of contamination introduced into the operating suite from outside. But the wheels of trolleys that are pushed through hospital corridors must also carry other bacteria, including staphylococci, into the operating suite. Viable counts of total organisms and of *Staph. aureus*, as well as *Cl. welchii*, are therefore relevant measurements in the assessment of contamination, though diluted by the effects of dispersal of these organisms from human sources in the operating suite.

These studies were made in ten hospitals and include comparisons of operating suites with and without clean zones and transfer areas. They showed that the wheels of trolleys used only in the operating suites ('theatre trolleys') usually had much lower levels of contamination with *Cl. welchii* than the wheels of trolleys used to convey patients from their wards to the operating suite ('hospital trolleys' and 'hospital-and-theatre trolleys'). It can therefore be assumed that fewer contaminants are introduced into the clean and probably also into the aseptic zone when there is a transfer area. However, in operating suites both with and without transfer areas the mean counts of *Cl. welchii* from samples taken in the clean zone and the theatre were significantly lower than those taken in the corridor. Counts of *Cl. welchii* from theatre floors in the suites with clean zones, with or without a transfer area, were very low, and there was no appreciable difference between suites with and without transfer areas judged by counts of *Staph. aureus* and of total organisms on theatre floors. Counts of airborne *Cl. welchii* fell within the same

range under both of these conditions, but were lower in theatres with a plenum ventilation system.

A theatre which had neither a transfer area nor a clean zone showed more *Cl. welchii* on the floor than theatres with a clean zone, whether a transfer area was present in the latter or not. It appeared that the clean zone might be an important factor in protecting the aseptic zone against contamination of floors with *Cl. welchii*. This protection in suites with clean zones may have been due to the exclusion of bacteria deposited from shoes when overshoes or theatre boots were worn by all of those who entered the theatre; it may also have been due, in part, to the presence of a longer stretch of floor between the hospital corridor and the aseptic zone, on which the more readily detachable bacteria could be deposited before the trolleys entered the theatre. The frequent washing of theatre floors is another factor which would tend to reduce the need for a transfer area; but in the progress from corridor to theatre the principal reduction in counts of *Cl. welchii* had occurred already in the clean zone, which is not washed as frequently as the theatre floor.

The bacteriological evidence suggests that, while it may be advantageous to have a clean zone, it is hard to justify the inclusion of a transfer area for trolleys in the theatre suite. This view is reinforced by the results of other studies in this laboratory which showed virtually no redispersal into the air of bacteria from floors on which they had recently settled, provided that brooms were not used for sweeping (Ayliffe *et al.* 1967). It may be thought desirable to exclude potentially contaminated trolleys from the theatre for operations on high risk cases. Transfer to the theatre table in the anaesthetic room could be arranged without a special transfer area.

Though *Cl. welchii* is a useful indicator of contamination from outside the operating suite, there is little evidence to suggest that gas gangrene occurs in operation wounds through contamination from the environment. Studies reported elsewhere on the isolation of *Cl. welchii* from the skin and on cases of post-operative gas gangrene show that self-infection is a much likelier mechanism of contamination in these cases (Ayliffe & Lowbury, 1969).

SUMMARY

The value of clean zones and of transfer areas in operating suites was assessed by comparisons of the amounts of contamination on floors, trolleys and footwear in suites with and without a clean zone and a transfer area; counts of *Clostridium welchii* were used as an index of bacterial contamination introduced into the aseptic zone from outside.

The mean counts of *Cl. welchii* on contact plates from the wheels of trolleys used to convey patients from wards to the operating suite ($67{\cdot}9 \pm 7{\cdot}68$ per plate) were significantly higher than those from theatre trolleys (i.e. those used only inside a theatre suite provided with a transfer area) ($3{\cdot}13 \pm 0{\cdot}47$ per plate); mean counts of total bacteria were only slightly lower on the wheels of theatre trolleys than on those of hospital trolleys. Other surfaces of hospital trolleys showed counts similar to those found on theatre trolleys.

Contact plates from floors showed significantly lower counts of *Cl. welchii* in the aseptic zone and the clean zone than in the hospital corridor, the protective zone and (when present) the transfer area.

The mean counts per 100 cm^2 of *Cl. welchii* were approximately the same on the floor of a theatre with a clean zone and a transfer area (0·83) as in one with a clean zone but no transfer area (0·5). Counts of total bacteria were higher in the latter. A suite with no clean zone or transfer area showed a higher mean count of *Cl. welchii* on contact plates from the aseptic zone (operating theatre) ($20·5 \pm 12·33$ per 100 cm^2). These higher levels of contamination were due to sporadic high counts of *Cl. welchii* found near the door of the theatre with no clean zone; in another theatre with no clean zone the level of *Cl. welchii* on the floor was not higher than that in the theatres with clean zones.

Theatres with plenum ventilation had lower mean counts of airborne *Cl. welchii* than those ventilated by windows: there was no significant difference in the levels of *Cl. welchii* on the floors of theatres with the two forms of ventilation.

On sampling with contact plates, theatre footwear yielded fewer total organisms, *Staphylococcus aureus* and *Cl. welchii* than outdoor shoes removed before entering the clean zone.

The hygienic value of transfer areas and clean zones is discussed. Bacteriological support could not be obtained for the former, but the latter appeared to contribute something to the cleanliness of the theatre by preventing heavy sporadic contamination.

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Bacterial contamination on the surface of hospital linen chutes

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Although linen disposal chutes have been used in hospitals for many years there has been a tendency to view them as a potential cross infection hazard. This suspicion, which in a few cases has led to their use being discontinued, may be dated from the publication of a report by Hurst, Grossman, Ingram & Lowe (1958). They regarded hospital linen and refuse chutes as a source of staphylococcal cross infection and showed that large amounts of air, heavily contaminated with *Staphylococcus aureus*, were being transferred to and from the chutes. This air movement may be brought about in two ways—the natural upward convection currents (stack effect) and the pumping or piston effect of material being dropped down the chute. Michaelsen (1963) demonstrated the beneficial effects of bagging linen and using an extract fan to ventilate the chute. He showed that these two precautions considerably reduced the airborne bacteria in the area of the chute. These measures along with good engineering construction, including reasonably airtight doors and a deceleration track, should contribute greatly to bringing airborne contamination to a satisfactory level. This is discussed more fully in our other reports (Reports, 1968).

One of the problems remaining is whether or not any facilities for chute cleaning should be provided in order that no significant contamination of the air is caused by bacteria on the inner chute surface. The aim of this study was to determine whether cleaning of the chute surfaces was necessary, and, if so, the frequency with which it should be done.

MATERIALS AND METHODS

Location and types of chutes studied

A total of 13 disposal chutes, located in eight hospitals, were studied. Given in Table 1 are those features of the 13 chutes which were considered likely to affect the amount of surface contamination on the inside of each chute. These were, the number of floors served by each chute, the hospital department and number of patients served by the chute, the method of disposal of the linen (in linen or polythene bags, or loose) and the arrangements for ventilating the chute. Apart from chute No. 9, which was plaster lined, and No. 8 which was 'Formica' lined the rest were made of metal. Both circular and rectangular chutes were included in the sample but the circular ones selected for this study were of large enough

diameter to allow samples to be taken of their internal surfaces using a Rodac plate. (Falcon Plastics, 5500 West 83rd Street, Los Angeles, 45, Calif. U.S.A.)

Only three of the chutes, all of which were used for the disposal of loose linen, employed a method of cleaning. Chute No. 1 was fumigated weekly with formaldehyde. Four pints of Liquor Formaldehyde B.P. was used. Exits and entrances were sealed and the length of time of fumigation was at least $8\frac{1}{2}$ hr. The volume of the chute was approximately 210 cubic feet. Chutes Nos. 12 and 13 incorporated a water flush. When this was operated from the basement, the chutes were washed down with fresh water.

Table 1. *General description of the chutes studied*

Hospital	Chute no.	No. of floors served*	Department served	Approx. no. of patients	Method of disposal	Chute vented†
A	1	6	Maternity wards and operating theatres	180	Loose	No
B	2	2	Wards	95	Linen bags	No
	3	2	Wards	80	Linen bags	No
C	4	4	Children's wards and operating theatres	100	Linen bags	Yes
D	5	5	Surgical wards	300	Linen bags	Yes
	6	5	Medical wards	270	Linen bags	Yes
E	7	1	Children's ward (infectious diseases)	25	Linen and polythene bags	No
F	8	6	General wards and operating theatres	300	Polythene bags	Yes
G	9	3	Neurosurgical wards	60	Linen bags	Yes
	10	1	Operating theatres	—	Linen bags	Yes
	11	1	Operating theatres	—	Linen bags	Yes
H‡	12	3	General wards	66	Loose	Yes
	13	3	General wards		Loose	Yes

* The number of floors served does not include the basement or exit floor.

† Vented by an opening at the top, either to outside or into roof space; none of the chutes had mechanical extract ventilation.

‡ Both chutes at Hospital H served the same area, no. 12 being used for pre-rinsed soiled linen, no. 13 for dry dirty linen.

Method of surface sampling

Rodac plates were used throughout this study (Hall & Hartnett, 1964). These disposable plates, $2\frac{1}{4}$ in. in diameter, were filled to the brim with agar medium and the agar allowed to set to form a meniscus on the uppermost surface. The lids were replaced and the plates incubated for 24 hr. at 37° C. to check their sterility before being stored at 4° C. In this series of experiments the plates were filled with 16 ml. of Blood Agar Base No. 2 (Oxoid Ltd)., usually containing 0·1 % phenolpthalein diphosphate penta Na salt. The phenolpthalein disphosphate was added as an aid in distinguishing colonies of *Staphylococcus aureus* (Barber & Kuper, 1951). When

sampling was carried out after formaldehyde sterilization 2 % sodium sulphite was added as a neutralizer to the medium. To take a sample, the agar meniscus was pressed against the surface under investigation. The Rodac plates were then incubated for 36 hr. at 37° C and counted. When plates with sodium sulphite in the media were used the incubation time was extended to 72 hr. Any colonies resembling *Staph. aureus* were tested for coagulase production and certain of these were phage typed. This was done by the standard set of phages supplied by the Staphylococcus Reference Laboratory, Colindale, London.

Number and type of surfaces sampled during the survey

In order to establish the amount of bacterial contamination present in disposal chutes, all eight hospitals mentioned previously were visited twice, with 1 month between each visit. Chute No. 1 was sampled the day before it was fumigated. Chutes Nos. 12 and 13 were sampled only once as a large amount of data concerning their surface contamination was being accumulated in another series of tests.

It was the aim at each visit to take four surface samples of the chute at each chute exit or entrance and at least one sample from the floor at each chute exit or entrance point. This was carried out throughout the survey with minor variations, e.g. no floor samples were taken at Chutes Nos. 1 and 4 as they were on the outside of the buildings, on a balcony.

The method of study of the build-up of bacteria on a clean duct surface

It was necessary as part of this study to establish the time for bacteria to build up on a linen chute which had been cleaned. This was carried out at chutes Nos. 1, 12 and 13. These were the three chutes which employed a method of cleaning, but no reliance was put on these methods. The following method was used instead.

Six pieces of polyvinyl material, adhesive on one side (Fablon), were attached to the inside surfaces of chutes Nos. 1, 12 and 13 at ground floor and basement levels. They were sterilized before and after attachment by washing down with 70 % ethanol in water. Each sheet was divided into a series of numbered sampling squares. Random number tables were used to group these squares into sets of six in order to ensure unbiased sampling. One set of six samples was taken, from each sheet after they had been fixed in position and sterilized. Samples were then taken in chutes Nos. 12 and 13 after 12 hr., 1 day, 2 days, 3 days, 6 days, etc., the final set being taken after 42 days. In the case of chute No. 1, samples were taken at 4, 14, 22, 39 and 46 hr. The practice of washing down chutes Nos. 12 and 13 with fresh water, which was described earlier, was discontinued during these tests. Chute No. 1 was sterilized with formaldehyde immediately before the start of the test.

The method of study of the efficiency of the two cleaning methods used

Fifty-two Rodac dishes on two occasions (a total of 104) were used to assess the cleaning efficiency of the water flushes on chutes 12 and 13. Samples were taken

immediately before and after this process. The efficiency of the formaldehyde sterilization method at chute No. 1 was tested once using 56 dishes. The positions sampled were as for the general survey.

RESULTS

Survey of bacterial contamination on the surface of linen disposal chutes

Table 2 shows the average bacterial count for each chute surface on the two visits, together with the mean count. These results are presented in the conventional manner as the number of bacterial colonies per Rodac plate. The chutes have been placed in the table in order of the amount of bacterial surface contamination.

Table 2. *The average test results, with the chutes ranked in order of bacterial surface contamination ('cleanliness'). All the counts are given as average numbers of bacterial colonies per Rodac plate.*

(Figures in parentheses thus ( ), give the average chute counts with the results from the sloping entry connexions omitted.)

Order of cleanliness	Chute no.	Average counts on the chute surfaces			Average floor count	% of *Staph. aureus* isolated from chute
		Test 1	Test 2	Mean		
1	11	3·3	1·0	2·1	5·3	0
2	10	5·1	1·8	3·4	12·3	2·0
3	9	6·3	2·7	4·5	107	0·4
4	4	3·8	11·7	7·7	—	0
5	8	6·8	10·6	8·7	125	0·6
6	7	7·2	11·8	9·5	88	0
7	6	8·1	19·2	13·6	93	1·1
8	2	13·7	21·4	17·5	109	0
9	1	28·6	26·9	27·7	—	0·1
		(9·6)	(14·3)	(12·4)		
10	3	12·3	47·8	30·1	169	0·2
11	5	39·2	32·9	36·1	164	4·0
12	12	41·5	—	41·5	422	0·5
		(19·7)		(19·7)	163*	
13	13	53·8	—	53·8	385	0·5
		(41·2)		(41·2)	126*	

* Average floor counts with the results from the basement floor omitted.

Chutes Nos. 1, 12 and 13 had sloping connexion pieces at each entry point, whereas the entry doors of the others opened directly on to the side of the chute. During the survey these sloping surfaces were sampled and it was found that they yielded counts which were about four times higher than those on the vertical surfaces. The average count in the three chutes concerned was 87·0 colonies per plate on the sloping surface compared to 22·0 colonies per plate on the vertical part of the chute. The results were therefore recalculated with the data from the sloping surfaces omitted and this is given in Table 2.

Table 2 also lists the average count on the floor outside each chute. These figures were calculated for bacterial counts of the floor surface at each entry and exit point. The average count on the floors over all the hospitals was 153 colonies

per Rodac plate. Only four of the individual floor counts deviated noticeably from this average. Two of these were in the operating theatres and in line with the lower counts found previously in operating theatre areas (Report, 1964). Two floor counts which were unusually high (422 and 385 colonies per Rodac plate) were at chutes Nos. 12 and 13. This was caused by an extremely high count on the basement floor, which was usually covered by loose linen (approximately 1400 colonies per Rodac dish in each case). Recalculation of the floor counts excluding these high basement floor counts gave average floor counts of 163 and 126 colonies per Rodac dish for chutes Nos. 12 and 13, respectively. Other chutes into which bagged linen was deposited showed no marked difference between the bacterial contamination on the basement or exit room and the other floors.

Calculation of the average bacterial count on the inner surfaces of all the chutes showed that the amount of contamination was eight times less than on the floor outside the chutes (19·7 as compared to 153 colonies per plate).

Number of Staphylococcus aureus *present in the linen chutes*

Table 2 also gives the percentage of *Staph. aureus* isolated in each linen chute, this being the average of both sampling visits. It can be seen from this table that the percentage of *Staph. aureus* present in the chute varied from 0 to 4·0 %, the average figure being 0·7 %.

Phage typing was carried out on some of the *Staph. aureus* isolated from chute No. 5. This was to establish if the high concentration obtained from this chute was caused by one or possibly two dispersers of staphylococci. However, the results showed that this was not the case but that the *Staph. aureus* isolated were made up of a large number of different phage types.

The build-up of bacteria on 'cleaned' linen chutes

Table 3 shows the build up of bacteria at the ground floor and basement levels of chutes Nos. 12 and 13 from the time the surface was sterilized until 6 weeks later. Table 4 shows the build up of bacteria at the ground floor and basement levels of chute No. 1 but with the sampling period confined to 46 hr.

It can be seen that the specially prepared surfaces were initially almost sterile, the highest concentration found being 1·8 colonies per Rodac plate. The results illustrate the well-defined phenomenon known as the 'plateau' effect. This is characterized by an initial increase in bacterial contamination on a sterile surface to a final constant concentration of contamination. This is caused by the bacteria which die being replaced by new ones; the higher the contamination rate the higher the 'plateau' would be. Although the 'plateau' concentrations as shown in Tables 3 and 4 tended to be slightly erratic (the higher concentrations probably coinciding with greater chute usage) it can be seen in chutes Nos. 12 and 13 that the 'plateau' had been reached in 24 and 12 hr. respectively. Extremely fast recontamination was also found at the basement position of chute No. 1, a mean concentration of 71·0 colonies per Rodac plate being reached within 4 hr. The fast recontamination of this chute was probably caused by the linen held back during the sterilization process being disposed off soon after the completion of the cycle.

At the first floor sampling positions of chute No. 1 it was found that there was little or no increase in the concentration of bacteria on the chute surfaces up till 46 hr. This chute was so constructed, that it was closed all the time except for the exit point and at the times when linen was being deposited. The whole chute, including exit and entrance, was attached to an external balcony. This meant that there would be very little, if any, temperature difference between the linen chute and the outside air and therefore very little ventilation caused by stack effect. The lack of ventilation of the chute was such that the smell of formaldehyde was

Table 3. *The build up of bacterial contamination on sterile surfaces of chutes Nos.* 12 *and* 13.

(Counts given as number of bacteria per Rodac plate.)

Elapsed time (days)	Chute No. 12		Chute No. 13	
	Basement	First floor	Basement	First floor
0	1·3	2·3	0·5	2·5
½	3·0	3·8	21·8	20·3
1	8·7	14·8	52·3	20·5
2	102·2	19·7	70·6	26·8
3	25·0	8·8	54·7	15·2
4	17·0	3·5	104·7	29·7
5	9·2	18·5	95·2	17·5
6	9·7	12·7	72·8	14·5
8	12·8	—	45·8	8·7
10	11·8	—	67·4	11·3
14	7·2	—	34·0	9·3
21	23·8	—	83·5	6·8
30	19·7	17·3	28·8	10·5
35	6·2	7·3	35·2	12·3
42	6·0	5·5	28·0	11·5

Table 4. *The build up of bacterial contamination on sterile surfaces of chute No.* 1

(Counts given as number of bacteria per Rodac plate.)

Elapsed time (hr.)	Basement	First floor
0	0	0·2
4	71·0	0·3
14	18·2	0·2
22	91·0	0·5
39	54·8	1·0
46	35·2	3·0

noticeable at the first floor entrance after a period of at least 46 hr. although the basement area where the chute stopped had cleared in less than an hour. This must be the explanation of the failure of the linen to recontaminate the first floor sampling position at the same time as the basement position was being recontaminated. Inspection of the results of sampling 6 days after sterilization showed that if the top floors had a lower concentration of surface contamination than the bottom floors, it was very insignificant.

Staphylococcus aureus were isolated in chute Nos. 12 and 13 but as they consisted of only 0·5 % of the total count in these chutes it necessarily meant that their isolation was intermittent. However, there was no indication of any build-up in these chutes. No *Staph. aureus* were isolated in chute No. 1.

The efficiency of the two cleaning methods used

The reduction in the number of bacteria sampled from the surface of the chute sterilized by formaldehyde was at least 98 %. Owing to the extra time required to sample and set up the experiment this test was carried out after 7 hr. of sterilization instead of the usual 8½ hr.

The results of the two sets of samples taken at the chutes which use a water flush showed that the efficiency of this process lay between 65 and 80 %.

DISCUSSION AND CONCLUSIONS

Eight hospitals were visited and thirteen linen disposal chutes sampled in order that the amount of bacterial contamination on the inside surface of these chutes could be determined. Although no two chutes were identical in those features which could have possibly influenced the surface contamination it was found that the amount of contamination in each chute was fairly similar, the mean surface bacterial count per Rodac plate varying from 2·1 to 53·8 with an average of 19·7. Even though the range of concentrations on the duct surfaces was small many of the chutes had features which helped to explain their high or low surface contamination. Of the three 'cleanest' chutes, two served operating theatres and were therefore used less, and the third had a porous surface which is known to cause low counts (Angelotti & Foter, 1958). The slightly elevated counts on the 'dirtiest' chutes were caused by sampling of the sloping entry connexion, which had four times more surface contamination than the vertical surface. This higher value may however be considered a true reflexion of the surface contamination, since the entry connexion was an integral part of this type of chute.

The surface contamination of the three chutes for which some means of cleaning was employed (a water flush for Nos. 12 and 13 and fumigation for No. 1) was much higher than might have been expected. These three chutes showed the highest, second highest and fifth highest amounts of surface contamination respectively. Four possible reasons can be thought of to fully or partially explain the high surface concentration in these chutes. These are: (a) The cleaning process was inefficient. (b) These were the only chutes down which loose linen was deposited. (c) These chutes all had sloping entry connexions which, as mentioned previously, had counts four times higher than those on the vertical surfaces. (d) The water flushes in chute Nos. 12 and 13 gave moist conditions suitable for the growth of bacteria.

It was shown, however, that the cleaning process was fairly efficient in chutes 12 and 13 (between 65 and 80 %) and highly efficient in chute No. 1 (at least 98 %). The possibility that water flushes in chutes 12 and 13 gave conditions suitable for growth of bacteria must be excluded as the considerable amount of hot air passing up the chute by stack effect ensured a rapid drying of the chutes. The average counts on the inner chute surfaces, with the samples taken on the sloping

surfaces omitted, are given in Table 2. It can be seen that this would have the effect of only slightly improving the relative positions of the three chutes.

It would therefore appear that the use of loose linen must have contributed largely to the high level of contamination of these three chutes, but that the methods of cleaning did not make them, at the time of sampling, any cleaner than the others. When cleaning was discontinued during the investigation of bacterial build-up in chutes Nos. 12 and 13 the surface contamination was found to be no higher than when they were regularly cleaned. The reason for this appears to be the rapid rate at which clean chutes become recontaminated. It was shown that the bacterial concentration on two chute surfaces studied (chutes Nos. 12 and 13) rose in 12 hr. in one chute and 24 hr. in the other, to a concentration which kept fairly steady for 6 weeks. In another chute (chute No. 1) a similar situation was demonstrated in which the bacterial concentration on the surface rose within 4 hr. to a concentration which showed no significant change after 1 week. However, at another sampling position in the same chute the rise was delayed at least 46 hr. It was assumed that the residual formaldehyde in this part of the chute was still exerting a lethal effect on the surface bacteria but there may have been some other cause such as the inability of sodium sulphite to neutralize the formaldehyde. However, the delay found in chute No. 1 would be of little significance as it could not be utilized in the normal hospital situation where chutes would be an integral part of the building. Apart from the difficulty of using such an irritant material, the normal 'stack' effect would ensure rapid elimination of the formaldehyde.

As *Staphylococcus aureus* is regarded as the organism most commonly associated with cross infection, the numbers present in the surface samples were determined. The number of *Staph. aureus* expressed as a percentage of the total sample varied between 0 and 4%, with an average of 0·7%. The 4% level of *Staph. aureus* occurred in only one chute, the remainder lay between 0 and 2%. However, as this was the only chute serving a block of surgical wards, this higher figure does not seem unreasonable. The average figure of 0·7% *Staph. aureus* is of the same order as that found on ward floors (Ayliffe *et al.* 1968). No evidence was obtained to suggest that there would be any build up of *Staph. aureus* in linen chutes.

Comparison of the amount of surface contamination on each chute with that of the floor outside the chute shows that the floor contamination was much higher. The over-all chute surface count was 19·7 colonies per plate, while the average floor count was 153—an eightfold difference. The floor samples appear to be typical of hospital floor samples but the chute samples are low in comparison to most hospital areas and would have been considered to be 'good' by standards laid down for ward floors. (Report, 1964; Pryor, Vesley, Shaffer & Walter, 1967). Although the release mechanism of bacteria from floors and chutes must be different, construction of linen chutes on the principles laid down in our previous reports (Reports, 1968) excludes the risk of cross infection from chutes except by a large source of airborne organisms within the chutes. Chute surfaces do not appear to fit into this category. It would therefore seem unnecessary to provide regular cleaning of the chute surfaces.

SUMMARY

A survey of 13 linen chutes in eight hospitals was carried out to assess the amount of bacterial contamination on the inner walls of these chutes. It was shown that the average bacterial count in these chutes was low by general hospital standards (19·7 bacterial colonies per Rodac plate). This concentration was eight times less than the average concentration found on the floor surface at each linen disposal and collection point (153 bacterial colonies per Rodac plate).

Three chutes sampled during the survey were periodically cleaned but appeared to derive only very limited benefits from the cleaning method they used.

Tests carried out on three linen chutes showed that the bacterial surface contamination of a sterilized chute could normally reach a maximum concentration in a period of between 4 and 24 hr.

It is concluded that so long as good engineering practices are used in the construction of linen chutes the bacterial contamination on their inner surface should not contribute to the problem of hospital cross infection. For this reason, and because of the impracticability of sterilizing linen chutes at very short intervals of time, it is considered that cleaning of chutes would serve no practical purpose.

Thanks must be recorded to the authorities of all those hospitals who gave their permission for us to study their linen disposal chutes and afforded us every assistance.

We should also like to thank Mr W. Carson, Leader of the Building Services Research Unit, for his help and encouragement.

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A mobile form of the Henderson apparatus

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INTRODUCTION

The apparatus described by Henderson (1952) has been used extensively to produce aerosols consisting of single bacterial cells for the purpose of studying respiratory infection. In the original apparatus fine droplets are generated by a Collison spray and mixed with the main air flow in a 'spray tube' in which air from a silica gel bed is passing at 20 litre/min. This produces a relative humidity around 50%. If the spray is fed from a bacterial suspension whose count is less than 10^9/ml. it is unlikely that any droplet will contain more than one organism. In addition each droplet residue will contain solid matter derived from the suspending fluid.

Over the past two decades various alterations have been made by the author to improve the instrumentation, stability and general ease of operation. The use of the apparatus as an aerosol generator for such devices as the Rotating Drum (Goldberg, Watkins, Boerke & Chatigney, 1958) led to a requirement for humidity control in the air issuing from the mixing tube. More recently the introduction of the microthread technique (May & Druett, 1968) has called for a mobile piece of equipment so that cloud production and microthread preparation can take place near to other special equipment such as chambers containing germicidal material (Druett & Packman, 1968). A block diagram of the apparatus developed is shown in Fig. 1 and a general view in Plate 1.

DESIGN CONSIDERATIONS

The apparatus was designed to operate at 20–95% R.H. at ambient temperature. The possible ambient temperatures considered were from 0 to 37°C. but it was assumed that temperature control, when required, would be provided by operating the mobile equipment in a temperature-controlled room.

The requirement of 20% R.H. at 0°C. made inevitable an increase in the flow rate through the mixing tube. Since the water content of the air at 0°C and 20% R.H. is 0·97 g./m^3 and a single jet Collison spray gives a nominal output of 0·77 g./min. of water, a flow rate of 66 l./min. is minimal. The higher flow rate is advantageous even at room temperature (20°C), as under these conditions it allows the use of a 3-jet Collison spray throughout the working range which increases the aerosol concentration and renders the apparatus less vulnerable to jet blockage.

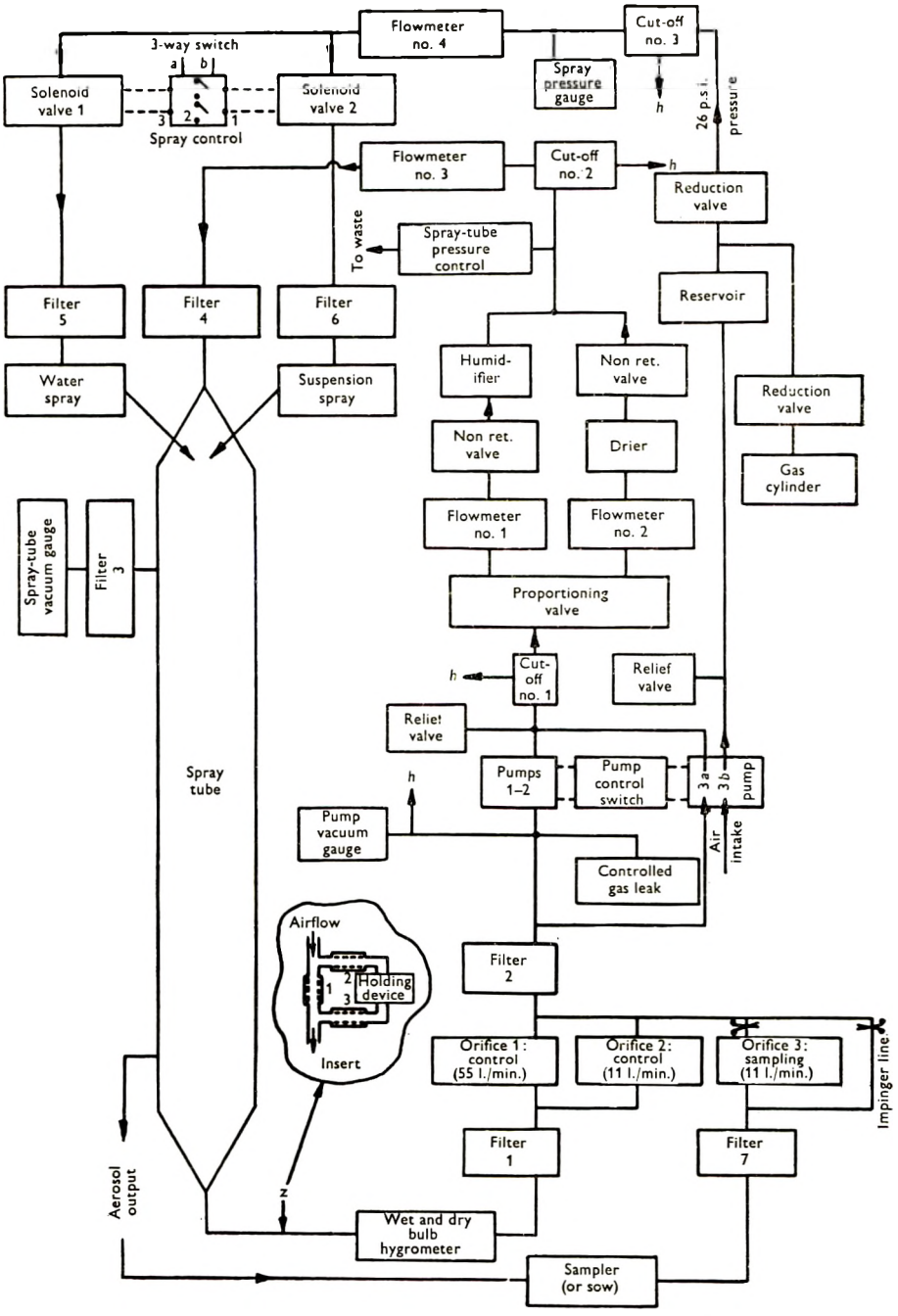

Fig. 1. Block diagram of the mobile Henderson apparatus.

Relative humidity control

The most convenient way to control relative humidity in a small dynamic cloud system is to mix, in known proportions, air containing water vapour at saturation ('wet air') with air which has passed through a silica gel bed ('dry air'). This is

achieved by passing the incoming air through a proportioning valve whose principle is shown in Fig 2. The air from the pumping system is led to the inlet channel in the sliding portion of the valve and passes thence through one of two slits into the appropriate outlet channel. The length $(a+b)$ of the inlet channel is equal to that of one slit (a) plus the length (b) of the solid land separating the two slits. With the slide in the extreme left hand position all the air is diverted into channel 1

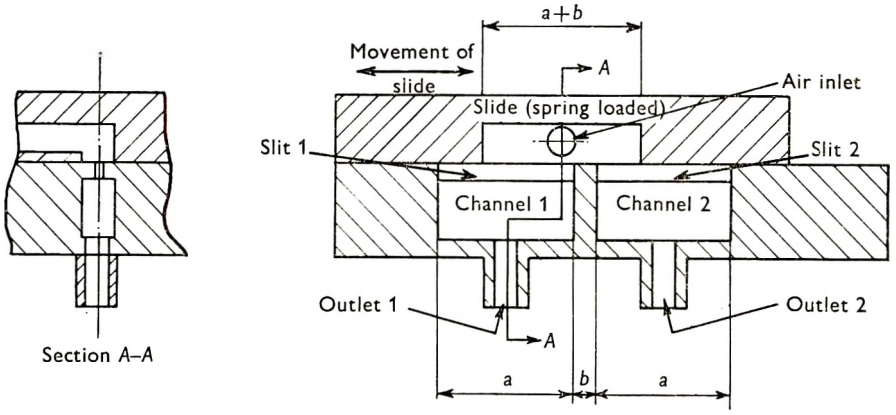

Fig. 2. Principle of the proportioning valve. In practice the slits of the proportioning valve are arranged to lie round a circle so that 'a' can be made longer without undue increase in bulk.

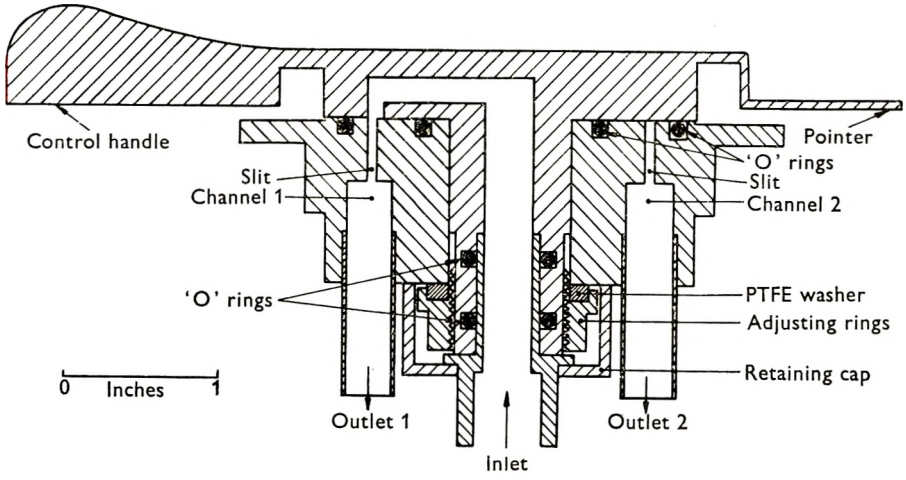

Fig. 3. Proportioning valve (drawn schematically).

and in the extreme right hand position it all passes to channel 2. At intermediate positions the air passes into both outlet channels proportionately to the length of the respective slits uncovered. In practice the slits are made as circular annuli and the general assembly is shown schematically in Fig 3. Provided that the pressure drop across the slits is large compared with that in the humidifier or drier, the total flow of air remains sensibly constant and the valve has a nearly linear characteristic. Air from the outlet channels passes to the appropriate flowmeters and thence to the humidifier and drier.

The drier

This consists of a 12 in. diameter × 18 in. high column of silica gel (or alumina) fitted with air spreading gauzes at the bottom.

The humidifier

In earlier models of this apparatus 'wet air' was produced by passing the airstream through a series of muslin screens dipped in water. This system has much to commend it: it is simple and has no moving parts and does not produce droplet residues in the airstream. The chief disadvantage was that the capillarity of the muslin deteriorated with age owing to the action of micro-organisms. This necessitated a periodic replacement of the muslin screens, which was time consuming.

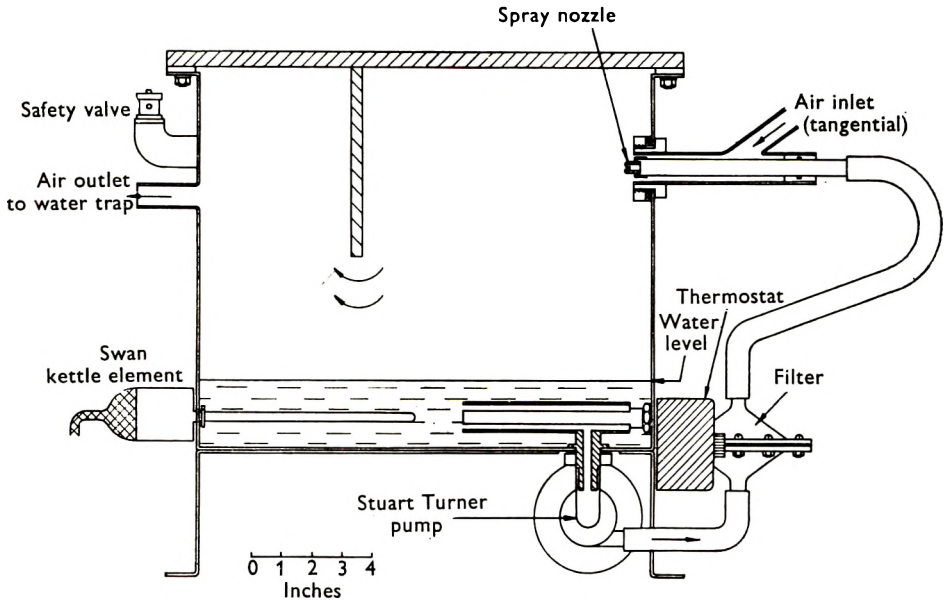

Fig. 4. Diagram of humidifier.

The more elaborate and expensive spray system was adopted, the general layout of which is shown in Fig. 4. Air enters at the right of the humidifier by means of a side tube on the spray inlet tube, offset to encourage turbulence, and passes into the main chamber through the water spray produced by a 726A Allman No. 1 nozzle. The unevaporated water falls to the bottom of the chamber and is recirculated to the spray by a Stuart Turner pump. Since the temperature of evaporating droplets falls rapidly to the wet bulb temperature of the surrounding air, it is difficult to achieve high relative humidity without prolonged contact of the air with sprayed droplets if the water is initially at the ambient temperature. By raising the temperature of the water in the chamber, and consequently of the air in contact with it, the same relative humidity can be achieved at a higher temperature. The subsequent cooling of the air mass in the circuit of the apparatus raises its relative humidity. We found that humidifier temperatures 10°C. above ambient were

adequate to produce 99 % R.H. in the air circulating in the apparatus and for most purposes lower temperature settings were adequate. It is essential that the humidifier temperatures should not be raised more than is necessary as this leads to the condensation of water in the subsequent circuit of the apparatus and a consequent loss of control. The required heating is supplied by a 'Swan' 1 kW. kettle element controlled by a Sunvic Thermostat. A baffle plate is inserted between the water spray and the outlet tube of the humidifier to reduce the carry-over of water droplets into the emergent airstream, and the air-flow subsequently passes through a droplet trap. Non-return valves made of plastic (La Bas Tube Co. Ltd.) are inserted in the humidifier and drier circuits to prevent diffusion of moisture from the former to the latter when the machine is not in use.

Supply of conditioned air to spray tube

Air in the appropriate proportions from the humidifier and drier is mixed, and passes via the spray tube pressure control and cut-off No 2 (Fig. 1) to flowmeter 3, and thence via filter 4 to the spray tube. The spray pressure control is a manually controlled gate valve which allows excess air to escape from the circuit. Cut-offs 1 and 2 are connected hydraulically to the vacuum side of the pumping system. They prevent air from entering the spray tube and driving the pressure positive should any fault develop in the low pressure section of the circuit (v. infra). They also ensure that the pressure in the spray tube falls when the pumps are started or stopped, a property which is important if pathogenic organisms are used. The general mechanical design of these hydraulic cut-offs is illustrated in Fig. 5.

Flowmeter 3 indicates the volume of air entering the spray tube. If the spray tube pressure control is fully closed the reading of flowmeter 3 should equal the sum of that shown by flowmeters 1 and 2. It thus serves the dual function of indicating leakage in the humidifier and drier circuit under test, and the correct functioning of the control orifices under running conditions. Filter 4 protects the humidification section from accidental contamination but being upstream of the spray tube it should not become contaminated under normal conditions.

Spray circuit

Compressed air for the sprays is supplied by one side of pump 3, which is a dual compressor unit. The other side of the pump unit is used to augment the capacity of pumps 1 and 2 (v. infra.) The compressed air passes through a reservoir which smooths out the pulsations in the circuit, and a reduction valve, and is delivered at 26 lb./in.^{2} to the spray circuit. As an alternative to the compressed air supply from pump 3, provision is made for the replacement of the air by other gases such as nitrogen from cylinders via a reduction valve as shown in Fig. 1.

Cut-off 3, which is hydraulically operated, interrupts the air-flow in the event of a failure in the low pressure section. Flowmeter 4 is specially calibrated to operate at 26 lb./in.^{2} and indicates the free air output of the sprays at their operating pressure. From the flowmeter the air passes through one of two identical alternative circuits consisting of a soleniod valve, an aerosol filter, and a spray head. One spray reservoir contains distilled water ('water spray') while the other is charged with a

suspension of micro-organisms ('suspension spray'). A 3-position switch selects one or other, or neither of these circuits. The power supply is derived from the output (*a*, *b*, Fig. 1) of the pump control switch and renders both sprays inoperative immediately in the event of an electrical supply failure. The water spray is used in 'conditioning' the apparatus before an experiment. It is also used when replacement air at the correct humidity is required by ancillary apparatus to compensate for the air removed by sampling. As the suspension and water head sprays are identical, and the solid content of the suspension does not materially affect the spray output, the change over from one to the other does not alter the relative humidity of the air in the spray tube.

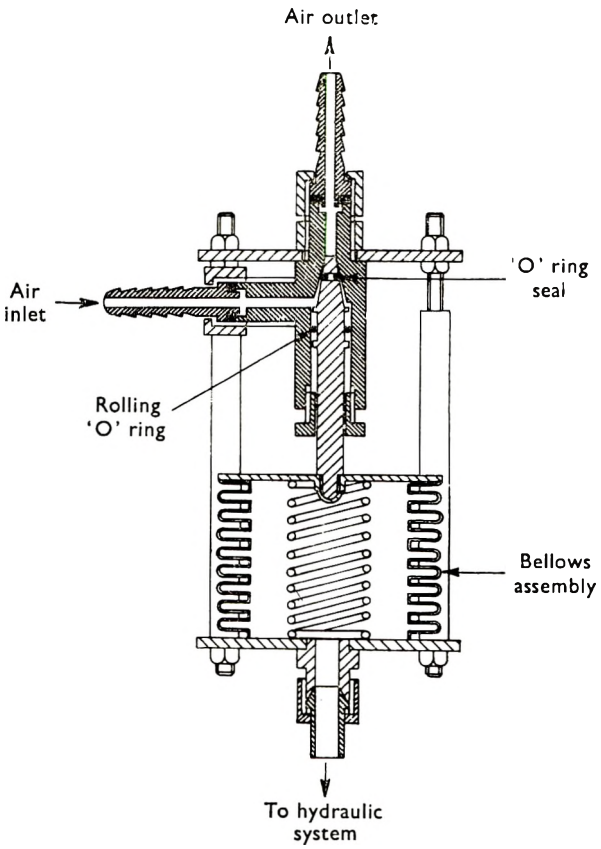

Fig. 5. Diagram of hydraulic cut-off.

The spray assembly

A general view of the spray assembly is shown in Fig. 6. All metal parts in contact with the suspension and the metal head (4) are made of stainless steel and the rest is of nickel-plated brass. Air at 26 lb./in.^{2} enters through the central tube (6) and passes into the spray head (7), the machining details of which are shown enlarged at the right of the figure. The rubber seal (9) prevents air leakage at this point. The microbial suspension (11) is entrained by the jet and issues as an aerosol (12). The droplet size of the aerosol generated is dependent not only on the spray

head dimensions, but also on the distance (1 in.) between the point at which the spray jet emerges and the glass wall (1) of the envelope containing the suspension, which acts as a baffle. These essential dimensions are the same as in the original

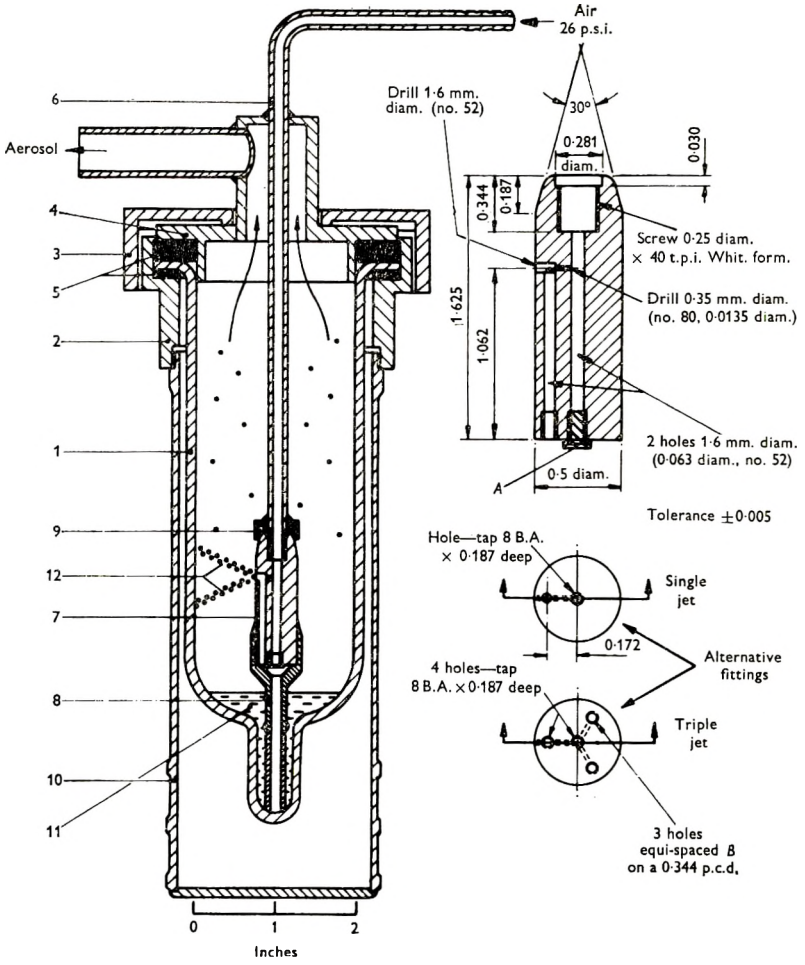

Fig. 6. Details of spray assembly.

Henderson apparatus. The glass envelope can be made in various forms according to experimental needs. The form shown is useful when only short bursts of spraying are needed, and the quantity of suspension available is restricted. For long-period spraying a test tube shape is more suitable, as it allows a greater volume of suspension to be used, and this reduces the slow upward drift in cell concentration in the suspension due to water loss by evaporation from droplets which are impacted on the glass wall and do not escape as aerosol. The sliding extension piece (8) to the spray head which terminates in a short length of plastic tube allows adjustment to various envelope designs. The lower free end of the plastic tube should be cut with a zig-zag edge, as a clean cut is liable to form a seal against the surface of the glass. The top surface of the glass envelope is ground flat and sealed against the

metal head (4) by the compressive action of two free threaded metal rings (2, 3) acting through rubber washers (5). The downward thrust of the upper metal ring (3) operates on a smaller radius than the upward thrust of the lower metal ring (2) and this permits a small amount of deviation from parallelism between the planes of the glass and metal without leakage. The outer metal casing (10) is useful in transport and can be left *in situ* during operation. The author prefers to remove it so that the operation of the spray jets can be watched during an experiment. The small machine screw marked '*A*' in the enlarged view of the spray head facilitates cleaning. The holes marked '*B*' are threaded so that they can be closed by a machine screw, reducing the number of jets in operation from 3 to 2 or 1 if required.

The spray tube

This consists of approximately 30 in. of 2 in. diam. tubing. The pipes through which the water and suspension spray enter terminate near the axis of the tube immediately in front of the tube carrying the main flow from filter 4. The spray tube can be separated into two halves for convenience in autoclaving. The distal end from the spray is fitted with a side tube through which aerosol can be extracted. If desired, ports for animal exposure can be added as described in Henderson's paper. For some bacterial suspensions sprayed into air at high relative humidity the spray tube length is inadequate for equilibrium to be attained between the droplet and the surrounding air. This is unimportant in microthread work or when holding apparatus, such as the Goldberg rotating drum, is to be used, since adequate time for equilibrium will be available subsequently. In inhalation studies the droplet size is of primary importance and special tube designs would be needed to ensure that equilibrium had been attained before inhalation.

To fill large holding devices which are being used repeatedly it is convenient to divert the main air flow of the apparatus at the point marked Z (Fig. 1) through a modification to the pipe work (see insert) and leave the holding device permanently attached. The portions of the tubing marked 1, 2, 3 are made of rubber. In normal operation 2 and 3 are closed by clips but 1 is left open. To divert the flow, clip 2 is released. Simultaneously 1 is closed and 3 opened. The flow now passes through the holding apparatus. After the appropriate time interval 1 is opened and 3 closed simultaneously, and subsequently 2 closed, trapping the aerosol in the holding device. The same process is used to remove the residual aerosol from the holding device at the end of the experiment, the Henderson apparatus being run with the suspension spray turned off for this purpose.

The hygrometer

On leaving the spray tube the air stream passes over a wet and dry bulb hygrometer and through filter 1. The hygrometer is placed upstream of the filter, as experience has shown that erroneous readings are obtained at high humidities if the filter precedes the hygrometer. Various forms of hygrometer have been tried but the wet and dry bulb system has been chosen since it is easy to sterilize by immersion of the contaminated parts in hypochlorite solution, and provided the 'wick' is replaced after each run it does not become 'poisoned' by deposition of the

passing aerosol. To obtain correct humidity readings the air flow over a standard hygrometer thermometer should not be less than 10 ft./sec.

Control orifices and filters

The flow in the spray tube is controlled by orifices 1 and 2 to a flow rate of 66 l./min. The flow rate of orifice 2 is chosen to match the flow rate which it is intended to use in the sampling circuit so that by opening one circuit and closing the other the flow through the spray tube is maintained at a constant value. The standard Porton impinger (Henderson, 1952) uses a flow rate of approximately 11 l./min. and acts as its own critical orifice. It is therefore convenient to set control orifice 2 to this value, and to install a sample orifice of the same value to control the flow through other sampling devices such as the 'Sow' (May & Druett, 1968). Orifices 1, 2 and 3 are of the Hartshorn type, the design of which has been described (Druett, 1955), and will give constant flow for pressure drops greater than 6 in. Hg. If a critical flow impinger is used in the sampling circuit, the pressure drop across the orifice must exceed 15 in. Hg. and owing to the limited capacity of the pumps this places a severe limitation on the amount of air or other gas which can be admitted at the controlled gas leak. For some purpose it may prove more convenient to use a separate external pumping system.

Filter 7 prevents contamination of orifice 3 and the pumping circuit. Filter 2 removes any penetration from filters 1 and 7 (which have a penetration of less than 0·002 % as determined by the sodium flame test) and provides second line protection to the pumps in the unlikely event of filters 1 or 7 failing.

The pump vacuum gauge and controlled gas leak

The pump vacuum gauge is calibrated in lb./in.^{2} and inches of mercury and indicates whether sufficient vacuum exists for the correct functioning of the control orifices (v. supra).

The controlled gas leak is provided at the pump intake to permit the steady changing of the air (or other gas) in the circuit. This is essential for oxygen replacement if animals are to be exposed: a small change is desirable in other cases so that any vapour released by a component cannot accumulate. Lengths of capillary tubing of appropriate bore form suitable orifices for this purpose.

OPERATION

Preliminary testing

Both sprays are charged with 20 ml. of sterile distilled water and connected into the circuit. The apparatus is brought into operation by pressing the pump control switch. The two spray heads are now tested. This is done by setting the spray control switch successively to positions 1 and 3 with the spray gauge reading adjusted to 26 lb./in.^{2} and taking readings from flowmeter 4. This should read 2·4 l./min. multiplied by the number of jets in use: a lower reading indicates jet blockage and a higher one leakage at one of the connexions or washers. At the same time a visual inspection of the spray emerging from the jet should be made as malfunction

due, for instance, to a blocked liquid feed tube can readily be detected. Blocked air jets can be remedied with a No. 80 drill: wire should not be used, as it scratches and distorts the bore of the air jet and prolonged use leads to erroneous flow readings. After both sprays have been checked the apparatus is switched off, the water removed from the suspension spray and the glass envelope (1) (Fig. 6) dried. It is not necessary to ensure complete sterility of the spray head, since the bacterial suspension added to it has a vastly higher bacterial count than any chance contaminant and the suspension is discarded after use so that no opportunity is afforded for the contaminant to grow in the suspending fluid.

The thermostat of the humidifier is adjusted to about 5°C. above the ambient temperature, and its heater and spraying unit switched on. About 15 min. should be allowed for thermal equilibrium to be established. After this period has elapsed the apparatus is started with the entry to the spray tube from the suspension spray closed and the water spray in operation. The spray tube pressure is adjusted to -1 in. water gauge by means of the spray tube pressure control. The humidity is adjusted by means of the proportioning valve until the appropriate value is obtained. An approximate setting can be obtained by calculation. If a relative humidity of 60% at 20°C. is required in the spray tube and flow meters 1 and 2 indicate together a total flow of 70 l./min., and an allowance of 6% R.H. is made for the water discharged from the water spray at this temperature, then flow meter 1 should be set to $[(60-6)\times 70]/100 = 37$ l./min. Further trimming adjustments are made as necessary.

Safety

Up to this point the apparatus has not been contaminated with micro-organisms and no safety hazard is involved. The appropriate safety precautions are now taken. While in normal use no aerosol escapes from the apparatus and every effort has been made to guard against the effects of electrical and mechanical failure, the 'second line of defence' recommended by Henderson of wearing respirator, hood, gown and gloves is adopted whenever pathogens are sprayed. Similar protection is afforded to the general population by ensuring that the room used is provided with an effective air extract system fitted with an efficient particulate filter. The use of chemical disinfectant aerosols advocated by Henderson is not favoured by the author since these act too slowly to provide any useful protection to the operator and, being for the most part persistant on surfaces, their vapour tends to find its way into apparatus and vitiate subsequent holding experiments. Strategically placed germicidal lamps, if well maintained, offer a more rapid safeguard should the need arise, and these can be run with the operator in the room, since he is fully covered.

The suspension spray

The appropriate bacterial suspension is placed in the reservoir of the suspension spray. The block on the entry to the spray tube is removed and the spray assembly connected to the apparatus. A check is made that the spray tube vacuum gauge is

reading − 1 in. water and that the hygrometer reading is correct. With full safety precautions in force the 3-way spray control switch is brought to position 1 and the bacterial aerosol is available for use.

Sterilization

The apparatus is arranged so that the contaminated portion (i.e. between filters 4, 5, 6 and filter 1) can be removed for autoclaving. The wet and dry bulb thermometers (which would be damaged by autoclaving) are placed in 5 % hypochlorite solution for 15 min. Alternatively a gaseous disinfectant can be used provided it is non-persistant. Pleated fibreglass paper filters will withstand autoclaving without breakdown but should be subject to periodic testing. Alternatively a jig type filter may be used which can be recharged after autoclaving.

CONCLUSION

This apparatus has been extensively used and found to be capable of running unattended for considerable periods. It can be moved from one site to another and be brought into operation within minutes. It has been transported on lorries and towed slowly for short distances behind vehicles on its own wheels and continued to function. Several variants of this apparatus have been built and formed the basic utility for the work described, *inter alia*, by Harper (1961), Harper & Hood (1962), Hood (1963), May & Druett (1968) and Druett & Packman (1968).

SUMMARY

A modification of the Henderson apparatus is described which is mobile and which can produce bacterial or viral aerosol in air whose relative humidity can be chosen in the range 20–95 %. The apparatus forms a basic utility for microthread, rotating drum or other aerosol studies.

Much help and advice has been given by Messrs. J. F. H. Peel and D. J. Garriock of the Engineering Section M.R.E. throughout the development of this apparatus. The proportioning valve is a modification of one originally used for a similar purpose by Mr K. R. May. Thanks are due to Messrs. G. J. Harper and A. M. Hood for criticism of the script.

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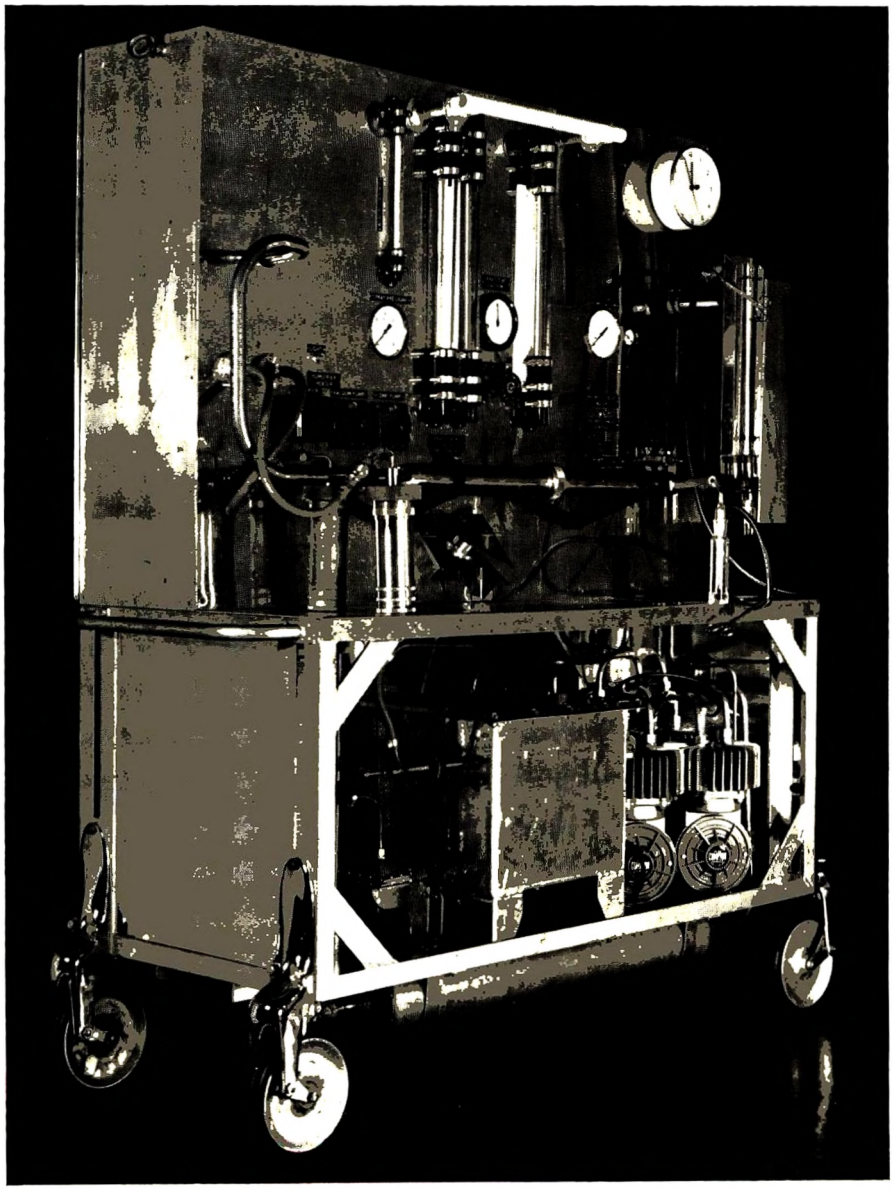
EXPLANATION OF PLATE 1

General view of the mobile Henderson apparatus.



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Dissemination and immunogenicity of live TRIC agent in baboons after parenteral injection

II. Experiments with a 'slow-killing' strain

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INTRODUCTION

During serial passage in chick embryos, some strains of trachoma/inclusion conjunctivitis (TRIC) agents undergo mutation and produce variants that, for a given dose, kill chick embryos more rapidly than the parent strain (Reeve & Taverne, 1963). Taverne, Blyth & Reeve (1964) termed these mutants *f*, or 'fast-killing', to distinguish them from the 'slow-killing' parent strains.

As part of a series of experiments with trachoma vaccines, we previously reported that the 'fast-killing' MRC-4 *f* strain of TRIC agent multiplied in baboons after parenteral injection (Collier & Smith, 1967). Subcutaneous injection was followed by multiplication in the skin and regional lymph nodes, and the agent was found in the spleen up to 11 days later. After intravenous injection, it was isolated from the blood up to 18 hr. later; the amount of infective agent in the spleen attained much higher levels and persisted longer than after subcutaneous injection, and in some animals there was good evidence of replication in peripheral lymph nodes. Twenty-four hours after intravenous (but not after subcutaneous) injection, TRIC agent was isolated from the liver. In line with earlier findings (Collier, 1961; Collier & Blyth, 1966), baboons injected intravenously were more resistant to challenge by the conjunctival route than were those inoculated subcutaneously.

In this laboratory, Blyth (1967*a*, *b*) found that the MRC-4 *f* strain was more virulent for guinea-pigs than the parent strain MRC-4 (full designation: TRIC/ /GB/MRC-4/ON) isolated from the eye of a newborn baby (Jones, 1961; Jones & Collier, 1962). After intracutaneous injection, it multiplied in the skin to higher titres and for longer periods than the parent strain, persisted longer in the regional nodes, and, unlike MRC-4, usually appeared in the spleen 3–10 days after injection.

The present paper describes an experiment to determine whether similar differences in the virulence of *s* and *f* strains could be established in baboons and, if so, whether such differences are related to immunogenicity; these questions are obviously important in choosing strains of TRIC agent for vaccine production.

MATERIALS AND METHODS

The procedures and materials were as previously described for our Experiment II (Collier & Smith, 1967) except that the vaccine was prepared from MRC-4.

General plan of experiment

On the first day (D 0) two groups each of 24 young baboons (*Papio cynocephalus*) were inoculated with a single 1·0 ml. dose of the same suspension of live MRC-4 vaccine. The animals of group A were injected subcutaneously into the medial aspect of the right upper arm, and those of group B intravenously into the femoral vein. A third group (C) of seven animals served as uninoculated controls. At intervals after injection (Tables 1 and 2), pairs of animals from groups A and B were killed and the amount of TRIC agent in various tissues was assayed by titration in chick embryo yolk sacs. In our previous experiment, TRIC agent was rarely detectable in small samples of the liver; this organ was therefore not tested. Titres were recorded in terms of $\log_{10}$ ELD 50 (50 % egg lethal dose) recovered from the skin lesion, group of lymph nodes, or whole spleen; the figures for the spleen were estimated from titrations of samples weighing approximately 1 g. The degree of immunity induced by the injections was estimated by comparing the resistance of representatives of the vaccinated and control groups to conjunctival challenge with the homologous strain.

Vaccine

This was prepared from the 9th chick embryo passage of strain MRC-4, partially purified from heavily infected yolk sacs that were harvested when 50 % of the inoculated chick embryos had died. Each sac was shaken for 2 min. at high speed with 5 ml. phosphate-buffered saline (Dulbecco & Vogt, 1954), strained through gauze to remove gross debris, and centrifuged at 2000 rev./min. for 15 min. in an angle head. The supernatant, constituting the 'vaccine' was stored in liquid nitrogen in 0·5 ml. amounts. Its titre was $10^{5\cdot8}$ ELD 50/ml.

Test for immunity

Eleven days after inoculation, the immunity of representatives of groups A and B was tested by inoculating their conjunctivae with the same suspension of MRC-4 used previously (Collier & Smith, 1967) and stored since then in liquid nitrogen. It was prepared from yolk sacs infected with the 8th chick embryo passage of this strain, and had a titre of $10^{5\cdot6}$ ELD 50/ml. The course of infection was compared with that in the control group C challenged similarly. The methods of scoring for severity of physical signs and presence of inclusion bodies were those of Collier & Blyth (1966).

RESULTS

Group A

Skin lesions. Within 48 hr. of subcutaneous injection, papular skin lesions 5–10 mm. in diameter appeared; as judged after dissection, necrosis was minimal. The lesions attained their maximum size on D 2 and regressed rapidly during the

first week (Table 1); by D 11 none were visible. Five minutes after inoculation, just over 10^4 ELD 50 of TRIC agent (i.e. 2 % of the amount inoculated) was recovered from the injection site in each of the two baboons examined at this time (Table 2). At 1 hr., $10^{3\cdot1}$ and $10^{5\cdot9}$ ELD 50 were recovered, although the latter figure is probably an overestimate; in this titration of skin suspension, the results

Table 1. *Weights of whole organs and tissue samples from baboons injected subcutaneously (group A)*

Days after injection	Baboon no.	Weights (g.) Skin lesion	Regional lymph nodes	Spleen
0 (5 min.)	407	0·1	NT	NT
	408	0·5		
0 (1 hr.)	409	0·3	0·6	NT
	410	0·1	0·7	
1 (18 hr.)	411	0·1	0·7	9·0
	412	0·1	0·4	7·7
2	413	0·9	0·8	11·8
	414	1·0	0·3	13·2
4	415	0·4	1·7	15·4
	416	0·1	0·7	9·6
7	417	0·1	2·1	12·2
	418	0·4	0·6	5·7
11	419	0·2	1·9	12·6
	420	0·1	0·6	9·1
15	421	0·4	0·5	8·0
	422	0·3	0·9	12·6
21	423	0·2	0·9	9·2
	424	0·2	0·9	7·5
28	425	0·2	1·2	7·2
	426	0·2	0·8	12·5

NT = Not tested.

Table 2. *Titres of TRIC agent in skin and regional lymph nodes of baboons injected subcutaneously (group A)*

Time after injection	Baboon no.	Skin lesion	Regional lymph nodes
D 0 (5 min.)	407	4·1*	NT
	408	4·2	NT
D 0 (1 hr.)	409	3·1	1·9
	410	5·9	< 1·2
D 1 (18 hr.)	411	< 1·9	< 0·8
	412	< 0·2	< 1·1
D 2 (42 hr.)	413	3·7	< 2·1
	414	3·2	< 1·7

* Infectivity titres expressed as $\log_{10}$ ELD 50 contained in the whole skin lesion or group of lymph nodes. NT = Not tested.

suggest carry-over of particulate material through the dilution series, with an abnormally high proportion of specific deaths at the higher dilutions. At 18 hr., no infective agent was isolated from either of the skin lesions tested. (In Tables 2 and 4, negative results are given in terms of the minimum infective titre detectable, taking into account the ratio of the weight of tissue inoculated into chick embryos to the total weight of the tissue or organ, and the lowest dilution tested.) The negative findings at 18 hr. are similar to those with MRC-4 *f* (Collier & Smith, 1967). Infective TRIC agent reappeared in the skin on D 2, but we were unable to detect it thereafter.

Regional lymph nodes. Apart from an occasional animal in whom the right axillary nodes appeared somewhat congested, the nodes draining the inoculation site remained virtually normal to the naked eye. Table 1 shows that in some animals increases in weight were observed from D 4 until the end of the experiment on D 28. Infective agent was recovered in low titre from the regional nodes of one animal an hour after injection, but could not be detected thereafter.

Spleen. In nearly half the animals examined there was an increase in spleen weight from D 2 onwards (Table 1); but TRIC agent could not be isolated from spleens tested at intervals from 18 hr. to 28 days after injection.

Blood. The agent was not isolated from blood samples taken 1 hr. and 18 hr. after subcutaneous injection.

Table 3. *Weights of whole organs and tissue samples from baboons injected intravenously (group B)*

Days after injection	Baboon no.	Weights (g.) Axillary lymph nodes	Spleen
1	391	0·5	9·2
(18 hr.)	392	0·6	10·1
2	393	0·6	7·3
	394	0·6	9·3
4	395	0·6	10·3
	396	0·8	11·4
7	397	0·6	9·8
	398	1·0	10·2
11	399	0·4	9·4
	400	1·0	14·0
15	401	1·0	15·8
	402	0·8	6·3
21	403	0·6	15·1
	404	1·1	11·9
28	405	1·0	11·8
	406	0·6	8·8

Group B

Lymph nodes. In about half the baboons examined from D 1 to D 28, the nodes in both axillae were hyperaemic. In several animals there was a moderate increase in the weight of this group of nodes from D 7 onwards (Table 3). TRIC agent could

not be isolated on first testing at 18 hr. (Table 4). It was detected in one of the pair examined at 42 hr., but all subsequent tests gave negative results.

Spleen. There was a moderate increase in average weight, first apparent on D11 and reaching a maximum on D21 (Table 3). TRIC agent could not be detected in spleens tested at 18 hr. On D2, but not thereafter, the agent was isolated from the spleens of both baboons tested (Table 4).

Blood. As with the animals inoculated subcutaneously, the agent was not detected in blood samples tested 1 hr. and 18 hr. after injection.

Table 4. *Titres of TRIC agent in spleen and peripheral lymph nodes of baboons injected intravenously (group B)*

Time after injection	Baboon no.	Lymph nodes	Spleen
D1	391	< 0·9*	< 2·3
(18 hr.)	392	< 1·0	< 2·4
D2	393	2·3	2·7
(42 hr.)	394	< 2·0	3·3

* Infectivity titres expressed as $\log_{10}$ ELD50 contained in the whole group of lymph nodes or whole spleen.

Table 5. *Response to conjunctival challenge in terms of individual and group scores*

Group	Baboon no.	Cumulative score at 28 days after challenge*	Mean score for group
A	435	37	
(subcutaneous)	436	48	
	437	21	
	438	25	34·0
	439	26	
	440	47	
B	429	23	
(intravenous)	430	30	
	431	50	
	432	40	31·0
	433	17	
	434	26	
C	441	45	
(controls)	442	45	
	443	62	
	444	40	43·1
	445	35	
	446	29	
	447	46	

* The individual cumulative scores are the sums of the scores recorded at four examinations made at weekly intervals after challenge.

Immunity to conjunctival challenge

Eleven days after parenteral injection the immunity of representatives of groups A and B was tested by comparing the severity of ophthalmic infection with that in control group C after conjunctival challenge with the homologous strain. Although the vaccinated animals had somewhat lower scores than the controls in terms of severity of physical signs and presence of inclusions in conjunctival scrapings (Table 5), analysis of variance showed that these differences were not statistically significant.

DISCUSSION

In our first paper on this subject (Collier & Smith, 1967) we described two experiments, the second of which dealt with the effects of single subcutaneous or intravenous injections of MRC-4 *f*, and is referred to in the Introduction. This experiment was closely comparable with that on the parent 'slow-killing' strains reported here, with one important difference to which attention must be drawn. Because of their ability to multiply to high titres in chick embryos 'fast-killing' strains such as MRC-4 *f* are potentially important in vaccine production; and the earlier investigation was specifically designed to determine whether a dose similar to that used for an actual vaccine would multiply in primate hosts after parenteral injection. The titre of the experimental MRC-4 *f* vaccine was $10^{7 \cdot 1}$ ELD50/ml.; but the vaccine made from MRC-4 contained 20 times less infective agent.

With this reservation, there was a pronounced difference between the behaviour of MRC-4 and its 'fast-killing' variant. After a subcutaneous dose, MRC-4 *f* multiplied to high titre in the injection site and could still be found there up to 3 weeks later; it also multiplied readily in the regional nodes, and appeared in the spleen 4–11 days after injection; by contrast, MRC-4 multiplied only to a limited extent in the skin, and could not be recovered after D2. That some multiplication did take place can be inferred from the loss of infectivity at 18 hr., and its reappearance at 42 hr.; the timing of these events, which were also observed with MRC-4 *f* in baboons, and with MRC-4 and MRC-4 *f* in guinea-pigs (Blyth, 1967*b*) accords with that of a single multiplication cycle. Unlike its 'fast-killing' variant, MRC-4 made only a transient appearance in the regional nodes, and failed to appear in the spleen. After intravenous injection MRC-4 was present in low titre in the peripheral nodes and spleen on D2 but not thereafter, again contrasting with MRC-4 *f*, which increased in the nodes up to 3 weeks after injection, and attained high titres in the spleen between D4 and D11. Although the effect of a smaller dose of infective elementary bodies cannot be altogether discounted, these differences in ability to multiply correspond closely with those in guinea-pigs, in which the doses of MRC-4 and MRC-4 *f* were more nearly comparable (Blyth, 1967*b*).

These findings may be related to immunogenicity; whereas single subcutaneous or intravenous injections of MRC-4 *f* induced good immunity to conjunctival challenge, its parent strain failed in this respect; nevertheless, we have previously shown that repeated injections of MRC-4 confer a high degree of immunity (Collier, 1961; Collier & Blyth, 1966).

Our results lend weight to the supposition that the immunogenicity of TRIC

agents depends greatly on the attainment of high dosage. Although such doses can be attained by use of a strain that multiplies within the recipient, or by repeated injections, it might be more satisfactory to look to the development of highly concentrated inactivated vaccines for use in man.

SUMMARY

After a single subcutaneous injection into baboons the MRC-4 strain of trachoma/inclusion conjunctivitis (TRIC) agent underwent limited multiplication at the injection site, but was then eliminated rapidly from the skin and regional lymph nodes. Forty-eight hours after a single intravenous injection, but not thereafter, it appeared in the peripheral lymph nodes and spleen. The single parenteral injections failed to immunize baboons against conjunctival challenge with the homologous strain. These findings contrasted with those previously reported for the more virulent mutant, MRC-4 *f*, which multiplied readily in the skin, lymph nodes and spleen, persisted in these tissues up to 3 weeks after injection, and conferred good immunity to conjunctival challenge with MRC-4. The difference in behaviour of MRC-4 and MRC-4 *f* might be accounted for, at least in part, by the use of a smaller inoculum of live MRC-4; but similar findings in guinea-pigs, reported elsewhere, suggest that the differences observed are real. In conjunction with previous work, the present study suggests that the immunogenicity of TRIC agent is closely related to the mass of antigen that can be administered to or propagated within the recipient.

We are grateful to Mr D. Venters for his able technical assistance.

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Strontium chloride and strontium selenite enrichment broth media in the isolation of *Salmonella*

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INTRODUCTION

Two enrichment methods, the tetrathionate broth of Müller (1923) and the selenite F medium developed by Leifson (1936), are widely used in the isolation of *Salmonella* from faeces and other contaminated materials. Modifications to these media have included the addition of selective inhibitory agents or growth factors to increase isolations both of numbers and of serotypes. However, certain serotypes, particularly *Salmonella choleraesuis*, frequently fail to grow in these media, and overgrowth by *Proteus* species and other enterobacteria interferes with the recovery of salmonellas. The enrichment culture method introduced by Rappaport, Konforti & Navon (1956), although an improvement on other methods in the recovery of most salmonellas, was found unsatisfactory for the isolation of *S. typhi*, and its efficiency in the recovery of *S. choleraesuis* from clinical materials had not been evaluated (Iveson & Kovacs, 1967; Anderson & Kennedy, 1965; Hooper & Jenkins, 1965).

Attention was directed to the extended use of specific ions incorporated in an enrichment medium which would be capable of recovering the full range of salmonella serotypes likely to be encountered in men and animals. Eisenburg (1918) had reported on the apparent resistance of *S. typhi* to strontium salts, and Hotchkiss (1923) had outlined the necessary concentrations of various salts, which included strontium chloride, required to inhibit the growth of *Escherichia coli* in peptone water. Rappaport & Konforti (1958) noted that the inhibitory action of malachite green could be selectively modified by certain bivalent cations to inhibit the growth of non-pathogenic Gram-negative microorganisms, while allowing the growth of certain salmonellas. More recently, Banič (1964) and Zajc-Satler & Banič (1965) used a selective combination of magnesium chloride and sodium hydrogen selenite. This enrichment medium, although efficient, was inferior to tetrathionate infusion broth.

A study was undertaken to test the selective action of strontium ion, in the form of the soluble chloride, and this was later expanded to include strontium selenite salt. Preliminary experiments were performed on pure cultures of *S. typhi*, *S. paratyphi A*, *S. choleraesuis*, *S. typhimurium*, *Arizona* spp., *E. coli*, *Pseudomonas* spp. and *Proteus* spp. On the basis of the results obtained, two simple and stable enrichment media were developed. These were used in parallel with the routine methods for salmonella isolation, as reported by Iveson & Kovacs (1967). The technique involved and the results obtained are presented in this report.

MATERIALS AND METHODS

The investigation comprised three complementary studies.

The first, Study I, involved the testing of 3,336 human faeces specimens and 128 sewage samples, using strontium chloride, selenite F and Rappaport enrichment broth media. Study II was directed particularly to the isolation of *S. choleraesuis* and involved the examination of 98 faeces specimens and 69 macerated gland samples from healthy pigs using the same enrichment media. Study III was undertaken to evaluate the recovery of *S. typhi* from 83 faeces specimens from suspect or actual cases of typhoid fever or carriers, and these were tested by the use of strontium selenite broth in addition to the media used in Studies I and II.

Study I

Faeces specimens were obtained from patients throughout Western Australia suffering from gastro-enteritis. Samples of 1–5 g. were transported to the laboratory in 1 oz. screwcap bottles containing 10 ml. Sachs (1939) enteric transport medium. Each faeces sample was mixed well and examined by inoculation of approximately 0·5 ml. into 10 ml. each of strontium chloride, selenite F and Rappaport enrichment broth media.

Sewage samples were collected by using swabs (Moore, Perry & Chard, 1952) which had been immersed for 24–48 hr. After lifting, each swab was placed in 100 ml. 1/4 Ringer solution and transported to the laboratory where, after mixing, 20 ml. from each sample was added to 150 ml. of each of the three enrichment media.

Study II

Inoculations of approximately 0·5–1 g. into 10 ml. each of the three enrichment media were made from faeces specimens and from macerated gland samples from healthy pigs.

Study III

Faeces from typhoid cases, carriers or suspect cases, emulsified in Sachs' transport medium, were inoculated in approximately 0·5 ml. amounts into 10 ml. each of the three media, strontium chloride, selenite F and Rappaport, and into the same volume of strontium selenite broth. On occasion, as additional controls, the tetrathionate broth of Preuss (1949) modified by Iveson & Kovacs (1967) and commercial selenite F, were also used.

All faeces samples were inoculated with pasteur pipettes of 2–3 mm. internal diameter. All specimens, in addition to being inoculated into the enrichment broths, were sown, in approximately 0·1 ml. amounts, on MacConkey, deoxycholate-citrate-agar (DC), Shigella and Salmonella agar (SS, Difco) and modified bismuth sulphite agar (BS) solid media. Sub-cultures from enrichment media were made at 18–20 hr. on SS and BS agar media, and again at 48 hr.

Media

Strontium chloride enrichment broth

Solution A (base)

Bacto tryptone (Difco)	0·5 g.
Sodium chloride	0·8 g.
Potassium dihydrogen phosphate	0·1 g.
Distilled water	100 ml.

Solution B (Stock)

Strontium chloride (B.D.H.)	60 g.
Distilled water	100 ml.

Solution C (Stock)

Malachite green (Merck)	0·4 g.
Distilled water	100 ml.

For use, to each 100 ml. solution A, 10 ml. solution B and 1·0 ml. solution C were added. 10 ml. volumes were distributed and sterilized by steaming for 30 min. The final pH was 5·0–5·5. The medium remained suitable for use after storage at room temperature for one month.

Strontium selenite enrichment broth

Preparation of strontium selenite. Twenty-five g. selenous acid (B.D.H.) were dissolved in 300 ml. distilled water. Strontium carbonate was added in approximately 1 g. amounts at a time with constant stirring at 50° C until effervescence ceased and a small amount of the carbonate remained undissolved. The pH at this stage was about 3·4. The mixture was vacuum filtered through a sintered glass disk (porosity 3) and added to approximately four times its volume of absolute ethanol with stirring. The amorphous salt precipitated and was collected by vacuum filtration on a sintered glass disk and washed twice with absolute ethanol. As much ethanol as possible was removed by vacuum from the precipitate on the sintered glass disk, which was then air dried in an oven at 50° C. The salt was soluble to approximately 2% by weight in water.

Preparation of strontium selenite enrichment broth:

Bacto tryptone	0·5 g.
Sodium chloride	0·8 g.
2% Strontium selenite	10 ml.
Potassium dihydrogen phosphate	0·1 g.
Distilled water	100 ml.

The broth was adjusted to pH 7·8, distributed in 10 ml. amounts and steamed for 30 min. The medium was found suitable for use after storage at room temperature for 2 months. Slight strontium phosphate precipitate was noted during storage but this did not interfere with its performance.

Other enrichment broth media

Selenite F medium used was that of Leifson (1936) and the Rappaport medium was modified by Iveson & Kovacs (1967).

RESULTS

In Study I a total of 475 salmonella strains were isolated from faeces, of which 474 were recovered by enrichment and 209 by direct culture methods. One strain was isolated only by direct plating, compared with 33 only by one or other of the enrichment media methods: 16 of these by Rappaport only, 12 by strontium chloride broth only and five by selenite F broth only.

The following *Salmonella* serotypes were recovered (the figures in parentheses indicate the frequency of isolations):

Salmonella typhimurium (221), *muenchen* (68), *chester* (21), *adelaide* (13), *oranienburg* (13), *wandsworth* (11), *senftenberg* (10), *newington* (9), *abony* (8), *anatum* (8), *bovis morbificans* (8), *orion* (8), *tennessee* (7), *onderstepoort* (6), *rubislaw* (6), *enteritidis* (4), *give* (4), *havana* (4), *hvittingfoss* (3), *ball* (2), *bredeney* (2), *brisbane* (2), *jangwani* (2), *lansing* (2), *saintpaul* (2), *eastbourne* (1), *emmasted* (1), *fremantle* (1), *kimberley* (1), *litchfield* (1), *ohlstedt* (1), *poona* (1), *potsdam* (1), *singapore* (1), *typhi* (1), *welikade* (1) and salmonella group (17), *Arizona* 26:26–25 (3), *Arizona* 5:29–30 (1).

Table 1. *Relative efficiency of strontium chloride, Rappaport and selenite F enrichment in the isolation of salmonella from* 3336 *human faeces and* 128 *sewage samples*

Combinations of enrichment media			Faeces		Sewage	
SC	R	SF	Salmonella positive	% efficiency	Salmonella positive	% efficiency
+	+	+	266	100	46	100
+	+	.	256	96·2	42	91·3
+	.	+	238	89·5	37	80·4
.	+	+	247	92·9	35	76·1
+	.	.	223	83·8	29	63·0
.	+	.	230	86·5	29	63·0
.	.	+	151	56·8	18	39·1

SC = strontium chloride; R = Rappaport; SF = selenite F.

With the exception of the *S. typhi* isolation, which was made from selenite F medium, no preferential isolation of a particular serotype was made by any one enrichment method.

Of the four *Arizona* isolations, one was positive on direct culture, two were made through selenite F, four through Rappaport and four through strontium chloride enrichment broths respectively.

Considering the 266 specimens which failed to yield salmonellas on direct plating, it was shown (Table 1) that 230 were positive using Rappaport's method compared with 223 and 151 using strontium chloride and selenite F enrichment broths respectively.

Of 128 samples of sewage examined, 46 yielded salmonellas. Of these, 11 were

isolated by strontium chloride only, nine by Rappaport only and four by selenite F only.

The following *Salmonella* serotypes were recovered from the sewage samples:

Salmonella adelaide (1), *anatum* (3), *bareilly* (1), *bovismorbificans* (1), *bredeney* (1), *chester* (13), *emmasted* (1), *give* (2), *heidelberg* (1), *meleagridis* (1), *muenchen* (13), *newington* (2), *oranienburg* (1), *orientalis* (1), *orion* (3), *potsdam* (1), *paratyphi B* (1), *rubislaw* (2), *senftenberg* (6), *singapore* (2), *saintpaul* (2), *typhi* (2), *typhimurium* (17) *wandsbeck* (1), *Arizona* 26:26–25 (1).

The two *S. typhi* isolations were made through selenite F medium and the single *Arizona* isolation through strontium chloride broth.

Of the 46 positive specimens, 29 isolations were made through strontium chloride, 29 through Rappaport and 18 through selenite F enrichment broth. Table 1 shows the combinations of enrichment culture methods yielding salmonellas from human faeces and sewage in Study I.

In Study II 27 isolations of salmonella were obtained from 98 samples of pig faeces; 24 were made through strontium chloride enrichment, 19 through Rappaport and 15 through selenite F. Of 69 pig gland samples examined, 15 yielded salmonellas, 11 of the isolations being made through strontium chloride, 10 through Rappaport and two through selenite F.

Table 2. *Relative efficiency of strontium chloride, Rappaport and selenite F enrichment in the isolation of salmonella from* 98 *pig faeces and* 69 *pig gland samples*

Combinations of enrichment media			Pig faeces		Pig glands	
SC	R	SF	Salmonella positive	% efficiency	Salmonella positive	% efficiency
+	+	+	27	100	15	100
+	+	.	26	96·3	15	100
+	.	+	26	96·3	11	73·3
.	+	+	21	77·8	10	63·3
+	.	.	24	88·9	11	73·3
.	+	.	19	70·4	10	63·3
.	.	+	15	55·6	2	13·3

SC = strontium chloride; R = Rappaport; SF = selenite F.

The following *Salmonella* serotypes were recovered from pig faeces and glands:

Salmonellae adelaide (3), *bahrenfeld* (2), *birkenhead* (1), *bovismorbificans* (4), *bredeney* (2), *chester* (3), *choleraesuis* (11), *derby* (9), *give* (1), *litchfield* (2), *muenchen* (4), *orion* (4), *saintpaul* (1), *tennessee* (1), *typhimurium* (6).

Of the 11 isolations of *S. choleraesuis*, four were made through both strontium chloride and Rappaport media, four through Rappaport only and three through strontium chloride only. Both direct and selenite F enrichment culture methods failed to yield *S. choleraesuis*.

The combinations of enrichment culture yielding salmonellas from pig glands and faeces are shown in Table 2.

In Study III four methods of enrichment culture as well as direct plating were

used in parallel on 83 faeces specimens suspected of containing *S. typhi* (Table 3). Forty-six of these specimens yielded *S. typhi* by one or more methods: 45 (97·8 %) were positive by strontium selenite, 35 (76·1 %) by selenite F, 25 (54·8 %) by strontium chloride, two (4·4 %) by Rappaport and 21 (45·7 %) by direct plating methods of culture respectively.

In a further comparative experiment 19 faeces specimens suspected of containing *S. typhi* were tested by the tetrathionate broth enrichment method in parallel with the four enrichment methods described, as well as by direct plating. All 19 proved positive through strontium selenite, 14 through selenite F, 11 through tetrathionate, eight through strontium chloride, one through Rappaport and five by direct plating.

Table 3. *Relative efficiency of strontium chloride, Rappaport, selenite F and strontium selenite alone or in a combination in the isolation of* S. typhi *from faeces of suspected typhoid fever cases or carriers*

	SC	R	SF	SX	SC.SF	SC.SX	SF.SX	Direct plating
No. positive	25	2	35	45	35	45	46	21
% efficiency	54·8	4·4	76·1	97·8	76·1	97·8	100·0	45·7

SC = strontium chloride; R = Rappaport; SF = selenite F; SX = strontium selenite.

In tests on 12 sewage samples known to contain *S. typhi*, all 12 were positive through strontium selenite but only seven were positive through selenite F enrichment methods. No specimen was positive on direct plating. These specimens were not tested by the other methods reported on in the study.

DISCUSSION

In a comparison of enrichment media for the isolation of *Salmonella* from faeces, Hobbs & Allison (1945) reported that selenite F medium was the most satisfactory method for the isolation of *S. typhi*. Recoveries of other serotypes, including *S. paratyphi B*, were comparable in both selenite and tetrathionate media. It was also reported that selenite F appeared suitable for the isolation of *Salmonella* from faeces specimens and lymph nodes of pigs, although occasionally both media might fail to suppress proteus species.

Similar results were reported by Cook, Frisby & Jebb (1951) on salmonella recoveries, which included isolations of *S. typhimurium*, *S. paratyphi B* and *S. typhi*, from tests on human faeces. Smith (1952, 1959) reported on a like experience in examinations of both human and animal specimens. Smith also noted that both selenite and tetrathionate media were unsuitable for the isolation of *S. choleraesuis*. Moore *et al.* (1952) reported improved isolations with selenite broth from sewage samples, and McCoy (1962) found that selenite and tetrathionate media were fully comparable and he recommended their use for sewage examination. He noted, however, that selenite favoured the isolation of *S. typhi*.

In trials of the enrichment broth introduced by Rappaport *et al.* (1956), Collard

& Unwin (1958), Hooper & Jenkins (1965) and Iveson & Kovacs (1967) achieved higher recoveries of salmonellas from faeces with Rappaport's medium than with either selenite or tetrathionate broths. However, the relative failure of Rappaport broth in the recovery of *S. typhi* placed severe limitations on its general application.

The results obtained in the present investigation further confirmed the relative superiority of Rappaport's enrichment broth over selenite medium in the recovery of *Salmonella* from both human faeces and sewage. At the same time, they showed that strontium chloride was comparable to Rappaport's medium in that, of the 266 possible recoveries of *Salmonella* from 3336 specimens of human faeces, where the organisms present were insufficient in number to provide direct culture positives, 230 and 223 isolations were respectively made through Rappaport alone and strontium chloride alone.

In the examination of 98 pig faeces and 69 pig glands, 26 and 11 isolations of *Salmonella* were made using strontium chloride and Rappaport medium in conjunction; the isolations through each medium alone were 24 and 11 for the strontium chloride and 19 and 10 for Rappaport, with the faeces and gland specimens respectively. Again, the relative poor showing for selenite F medium was noteworthy.

The 11 isolations of *S. choleraesuis* were made through Rappaport and strontium chloride media alone or in combination, while the complete failure of selenite F in the isolation of this serotype would preclude its use in the sphere of public health and veterinary bacteriology.

In the third trial in the present investigation (Study III), where the recovery of *S. typhi* from the faeces of typhoid fever cases and carriers by the use of selenite F, Rappaport and strontium chloride and strontium selenite enrichment media were under test, it was shown that, with the combined use of strontium selenite and selenite F, no specimen found to contain *S. typhi* by any combination of methods failed to yield the organism. It was also shown that strontium selenite alone failed in the isolation of *S. typhi* from only one of the 46 positive faeces as opposed to 11 failures with selenite F, 21 failures with strontium chloride, 44 failures with Rappaport and 25 failures on direct plating.

In the limited trial with 19 faeces suspected of containing *S. typhi*, tetrathionate broth was instrumental in the recovery from only 14, compared with all 19 by strontium selenite medium.

The isolation of *S. typhi* through strontium selenite from all 12 sewage specimens examined in this study, as compared to seven isolations through selenite F under parallel conditions, provided further evidence of the superiority of strontium selenite over other enrichment broth media for the recovery of *S. typhi* from human faecal matter.

SUMMARY

Two new media, strontium chloride and strontium selenite broths, are described for the enrichment culture of *Salmonella* from human and animal material.

In comparative trials, strontium chloride was found to be comparable to Rappaport medium for the recovery of a wide range of *Salmonella* serotypes from

human faeces and sewage, and from pig faeces and glands. Both were superior to selenite F medium.

Strontium selenite was found to be superior to selenite F in the recovery of *S. typhi* from human faeces and sewage.

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Some experiments relating to artificial immunity in enzootic pneumonia of pigs

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Enzootic pneumonia of pigs is a common disease with a world-wide distribution. In Britain, the majority of pig herds appear to be infected, and the resulting economic loss is very great. Under intensive husbandry conditions, where large numbers of pigs are closely confined in a common air-space, the pneumonia is slow to heal and commonly persists to the normal slaughter age and beyond. Pigs so affected require significantly more food—often an average increase of 20 to 25 %—to make the same live-weight gain.

Because there is no satisfactory way of combating the economic effect of this disease in an infected herd, except by reducing the concentration of animals, most efforts to date have been spent on the problems of eradication. A co-ordinated health-control scheme was initiated in this country for enzootic-pneumonia-free herds in 1959 (Goodwin & Whittlestone, 1960) but various difficulties have arisen, the greatest being the problem created by the high reinfection rate (Goodwin & Whittlestone, 1967). Some alternative or complementary method of control would be very useful, therefore, and the immunological approach is an obvious choice.

There is good reason to believe that a strong natural immunity occurs in the field, and this is supported by the findings of Lannek & Börnfors (1957) who were unable to reinfect pigs which had recovered from the experimentally-induced disease. More recently, Goodwin, Hodgson, Whittlestone & Woodhams (1969) infected hysterectomy-produced, colostrum-deprived pigs with enzootic pneumonia and then challenged them, after recovery, with the same strain of the disease. These animals developed virtually no lung lesions when inoculated with lung suspensions that produced extensive lesions of enzootic pneumonia in control animals; this was so, even when the pigs were as young as 16 days old when first infected and were not challenged until up to 60 weeks later.

Goodwin, Pomeroy & Whittlestone (1965, 1967) established that enzootic pneumonia was caused by a mycoplasma, by inducing the disease with colonies that had been passaged on solid medium; these colonies were named *Mycoplasma suipneumoniae*. Consequently, the way has been cleared to investigate the possibility of inducing an artificial immunity to this disease, using pure cultures of the causal agent. The present paper describes some preliminary experiments in this direction.

MATERIALS AND METHODS

Antigens

The J strain of *M. suipneumoniae* and strain 603 of *Mycoplasma hyorhinis* were used (Goodwin *et al.* 1967).

The cloned strains of these two organisms had passed through five consecutive single-colony subcultures on solid medium.

The *M. suipneumoniae* antigen that was injected into pigs was uncloned and was grown in the liquid medium previously described (Goodwin *et al.* 1969). Each batch of this antigen was the fifth passage from a culture which had induced enzootic pneumonia after being passaged considerably more times in parallel. The antigen was washed three times by being shaken in phosphate-buffered saline (PBS) and then centrifuged for $\frac{1}{2}$–1 hr. at 30,000–50,000 ***g***. on each occasion. The antigen was then resuspended in PBS and the opacity compared with Brown's tubes: on the assumption that two mycoplasma organisms would be equivalent in opacity to one organism of *Brucella abortus*, the antigen suspensions used (before mixing with adjuvant) contained about 10^{11} mycoplasmas/ml. Formalinization was with 1/2000 formaldehyde at 37° C. for 6 hrs. When Freund's complete adjuvant was used, this was mixed with approximately equal quantities of antigen suspension containing 200 units of penicillin and polymyxin and 240 μg. of streptomycin/ml. All the pigs in Table 1 received 1–2 ml./dose of either antigen suspension alone or of antigen suspension plus adjuvant; the sow in Table 3 received 5 ml. doses.

Serological techniques

Serum samples were stored at about −20° C.

Metabolic inhibition (MI)

This test was performed as previously described (Goodwin *et al.* 1969), using both heated (56° C. for 30 min.) and unheated serum samples, and both cloned and uncloned strains of *M. suipneumoniae*.

Indirect (passive) haemagglutination (IHA)

This test was performed as before (Goodwin *et al.* 1969).

Complement fixation (CF)

This test was carried out in the same general manner as before (Goodwin *et al.* 1969), but with the following variations. The 4 % red-cell suspensions were standardized colorimetrically in a spectrophotometer. Only one series of doubling dilutions was made, starting at 1/10, and the test was discarded if the titre of the standard positive pig serum differed by more than one dilution from the usual titre. The titres quoted represent fixation of 50 % or more.

As mentioned in our previous paper (Goodwin *et al.* 1969) this test presents various difficulties. Inactivated (56° C. for 30 min.) and unheated samples of some sera were titrated in parallel: in some cases, the titres were the same but, in others, the fixation in the tests with heated serum was reduced to 50–75 % throughout the

dilution range. This suggested that inactivation damaged some stabilizing accessory factor. The short period of fixation (3 hr. at room temperature) used by Roberts (1968) was found to be unsatisfactory, in that false positives and cross reactions, as between *M. suipneumoniae* and *M. hyorhinis*, for example, were obtained: such discrepancies did not seem to occur with overnight fixation.

Precipitation in agar-gel

The double-diffusion method of Ouchterlony (1964) was used. The *M. suipneumoniae* and *M. hyorhinis* antigens were prepared as for the IHA and CF tests.

Pigs

The pigs listed in Tables 1 and 2 were all hysterectomy-produced, colostrum-deprived (HPCD) animals. Litter 183 was 14 weeks old and litter 185 was 5 weeks old when first injected. Litter 186 (Table 2) was 17 days younger than litter 185. The sow shown in Table 3 came from a herd established entirely from HPCD pigs and maintained in isolation on the Veterinary School farm: the sow was 18 months old when first injected and had had one litter previously. The lungs of routinely slaughtered pigs from this herd are regularly checked and have been free from lesions of enzootic pneumonia.

All the pig experiments were peformed in a specially designed isolation building, as previously described (Goodwin, Pomeroy & Whittlestone, 1968). The injection routes for the antigen are given in the text. Challenge was by intranasal inoculation of suspensions in broth of ground lung affected with the J strain of enzootic pneumonia. This strain has been repeatedly used to infect pigs and the only mycoplasma isolated from it has been *M. suipneumoniae*. The challenged pigs in Table 1 received about 5 ml. of a 1/10 lung suspension. This dose became the high dose in Table 2, where the pigs receiving the lower dose were given 5 ml. of a 1/100 suspension. The doses of lung suspension used in the sow-and-litter experiment are detailed in Table 3.

The criteria for diagnosing enzootic pneumonia were as previously described (Goodwin *et al.* 1969). In some cases, the diagnosis was confirmed by isolating and identifying *M. suipneumoniae*. The scoring system for recording the extent of the consolidated lesions was related to the fact that, in enzootic pneumonia, such lesions occur almost entirely in the apical and cardiac lobes of the lung, in the intermediate lobe, and in the leading edges of the diaphragmatic lobes. Ten points were allocated to each apical or cardiac lobe, five points to the intermediate lobe and five points to each leading edge of the diaphragmatic lobes; thus, if all this tissue were totally consolidated (which would be an unusually severe case) the pneumonic score would be 55. The mycoplasma score was based on the numbers of mycoplasmas with the morphology of *M. suipneumoniae* seen in touch preparations made from pneumonic tissue: 0 = none; 1 = few; 2 = moderate numbers; 3 = large numbers; and 4 = very large numbers.

RESULTS

Stimulation of antibodies in young pigs with formalinized antigen, and the subsequent challenge of these animals

Eleven pigs were injected twice with formalinized antigen, the first injections being given with Freund's complete adjuvant. They were injected intramuscularly on each occasion, or intradermally followed by intravenously, and the interval between the two injections was 18 days. The IHA and CF titres of the sera are shown in Table 1, together with the results of challenging six of these animals plus two controls.

The pigs that had been injected with antigen showed no obvious resistance to challenge. One of the two positive controls (3013) developed only a small area of enzootic pneumonia, as did one of the injected pigs (3016). The average pneumonic score and the average mycoplasma score in these controls was 14 and 2·5, respectively; the comparable figures for the six injected pigs were very similar (12 and 2). Table 1 shows that neither the IHA nor the CF serum titre appeared to be related to protection, in that the two injected pigs with the smallest area of pneumonia had the highest and lowest CF titres, while the pig with the highest IHA titre had almost as much pneumonia as the pig with the lowest IHA titre.

The IHA titres developed slowly, 4 of the 11 still being < 10 by 18 days after the first injection; however, substantial titres were recorded by 14 days after the second injection, five of them being over 20,000.

The CF titres appeared earlier than the IHA titres, in that all but one were 80 or more by 18 days after the first injection. However, they did not increase so dramatically thereafter: the mean CF titre after the first injection was a little over 200, and the mean titre after the second injection was about 930.

No significant change in the CF titres had occurred by 19 days after challenge, but there was a notable increase in most of the IHA titres by this time.

Effect of a lower challenge dose

The second part of this main animal experiment involved the five remaining injected pigs. It seemed possible that some degree of immunity might have been induced but that this had been overwhelmed by a too heavy dose of challenge inoculum. The effect of a smaller challenge was therefore observed in pigs 3031, 3032 and 3030 (Table 2) for which pigs 3027, 3028 and 3029 served as positive controls. The latter three controls all developed lesions of enzootic pneumonia, as did one of the injected pigs (3032); the other two injected pigs (3031, 3030), however, did not develop lesions and no mycoplasmas were found in touch preparations made from the lungs. It is very unusual to obtain complete negatives in such HPCD pigs, and this result might therefore indicate that a sufficient degree of protection had been induced by the injections. These two pigs were also the ones with the highest IHA titres and the ones that had received the double intramuscular injections; one or both of these facts might be relevant to their lack of lesions.

Table 1. *IHA and CF titres developed by pigs injected with formalinized suspensions of* M. suipneumoniae, *and the effect of challenging such animals with enzootic pneumonia*

Pig	Litter	Route of injection	Serum titres (reciprocals) 18 days after 1st injection (and just before 2nd injection) IHA	CF	14 days after 2nd injection IHA	CF	37 days after 2nd injection (when challenged) IHA	CF	56 days after 2nd injection (when killed) IHA	CF	Pneumonic score*	Mycoplasma score*
3017	183	I/M; I/M	160	80	640	640	1280	640	5120	640	12·5	3
3019			< 10	160	80	640	320	640	10,240	1280	22	3
3031			80	160	2560	2560	ND	ND	Not killed		NA	NA
3065			40	20	1280	640	ND	ND	Not killed		NA	NA
3016		I/D; I/V	160	640	20,480	1280	10,240	1280	20,480	1280	1	1
3018			20	640	40,960	1280	10,240	640	81,920	1280	18	2
3032			20	80	640	1280	ND	ND	Not killed		NA	NA
3066			< 10	160	2560	320	ND	ND	Not killed		NA	NA
3014	185	I/M; I/M	40	80	20,480	640	10,240	320	40,960	320	3	1
3015			< 5	160	40,960	320	40,960	320	40,960	640	17	2
3030			< 5	160	40,960	640	ND	ND	Not killed		NA	NA
3012	183	—	—	—	—	—	Uninjected positive controls challenged on same day		< 5	40	26	2
3013									< 5	40	2	3

Note. The 11 injected pigs were all bled before their first injection: the IHA titres were all < 5, and the CF titres were all < 10.
* The scoring systems are described in Materials and Methods.
ND = not done; NA = not applicable; I/M = intramuscular; I/D = intradermal; I/V = intravenous.

Table 2. *Low-level challenge of pigs that had previously been injected with formalinized suspensions of* M. suipneumoniae, *and a high-level challenge of pigs that had probably recovered from enzootic pneumonia*

Pig	Litter	Route of injection	Dose of first challenge inoculum	Serum titres (reciprocals)								Pneumonic score*	Mycoplasma score*
				Day before challenge		22 days after challenge (when killed)		17 weeks after challenge (when rechallenged)		26 days later (at slaughter)			
				IHA	CF	IHA	CF	IHA	CF	IHA	CF		
3030	185	I/M; I/M	Low (5 ml. of 1/100)	81,920	320	81,920	320	—	—	—	—	0	0
3031	183	I/M; I/M		2560	640	81,920	1280	—	—	—	—	0	0
3032		I/D; I/V		640	640	40,960	640	—	—	—	—	23	2
3027	186	None;		< 5	< 10	< 5	40	—	—	—	—	8	2
3028		Positive		< 5	< 10	< 5	40	—	—	—	—	3·5	2
3029		controls		< 5	< 10	< 5	80	—	—	—	—	8·5	2
3065	183	I/M; I/M	High (5 ml. of 1/10)	1280	640	—	—	20,480	1280	10,240	1280	1	0
3066		I/D; I/V		5120	320	—	—	20,480	640	10,240	320	0·5	0
						20 days after challenge (when killed)							
3022	186	None;		< 5	< 10	< 10	40	—	—	—	—	4	2
3023		positive controls		< 5	< 10	< 5	40	—	—	—	—	7	2
								At challenge					
3064	183	None; positive control		—	—	—	—	< 5	< 10	20	80	8	3

* The scoring systems are described in Materials and Methods.

Response to a superimposed experimental infection

In parallel with the above low-dose challenge experiment, two pigs (3065, 3066) were given the higher challenge dose used earlier (Table 1) with pigs 3022 and 3023 serving as positive controls. The control animals were killed 20 days later to check that the inoculum was capable of inducing enzootic pneumonia and as this was the case, pigs 3065 and 3066 were kept alive, in the hope that they would have already developed enzootic pneumonia, as did their litter-mates (3017, 3019, 3016, 3018) when similarly challenged earlier (Table 1), and thereafter become naturally immune during recovery. At 17 weeks after their first challenge, therefore, they were rechallenged with the higher dose of infective inoculum and killed 26 days later. Both pigs had only very small lesions of a type resembling late enzootic pneumonia and no mycoplasmas were found in the touch preparations, whereas the positive control (3064), challenged and killed at the same time, had active lesions of enzootic pneumonia and large numbers of organisms with the morphology of *M. suipneumoniae* were present in the touch preparations. It seemed from this that both pigs 3065 and 3066 had strongly resisted their second challenge, almost certainly because they had developed a natural immunity: this immunity, however, was not associated with IHA or CF serum titres that were any higher than those of pigs 3018 and 3015 (Table 1) which did not resist a comparable challenge.

Metabolic-inhibition test

While this work was in progress, it had become apparent in other work (Goodwin *et al.* 1969) that the MI test might be of little value, because non-specific inhibitory substances were present in the sera of some pigs both before and after experimental infection; this non-specific inhibition was sometimes reduced by heating the sera (56° C. for 30 min.), but at other times it was not. Only a limited number (37) of serum samples were examined with this test, therefore, in order to see whether the MI test might be more helpful with serum samples from pigs that had been injected with antigen. The results are not presented in detail; they can be summarized, however, as follows.

Unlike some of the sera in our previous study (Goodwin *et al.* 1969) all the pre-exposure serum samples that were examined had MI titres of < 3. After the pigs had been injected with antigen, however, the sera became positive: the highest titre in the heated serum samples was 1/12 and the highest in the unheated samples was 1/24. Titres of this order, however, were not associated with obvious immunity and there was no increase in the MI titres of the heated serum samples from pigs 3065 and 3066 (Table 2) when these animals became immune following natural infection.

Precipitation in agar-gel

Sera from the 11 pigs in Table 1 that were injected with antigen were examined by this method against *M. suipneumoniae*. All were negative before injection, 9 out of 11 were positive 18 days after the first injection and all were positive 14 days after the second injection; the longest period between the first injection of antigen and challenge was $11\frac{1}{2}$ weeks and all the five pigs examined after this time were

positive by the agar-gel precipitation test. The sera of two pigs killed 22 and 26 days after infection were negative.

The positive sera usually gave two or three precipitation lines by this method, all of which were continuous with the lines obtained with the serum from a rabbit which had been injected with the same antigen. One pig serum gave a fourth line. No precipitation lines were obtained when these sera were tested against *M. hyorhinis*.

The sera and the colostrum sample from the sow and the sera from the piglets (Table 3) were also examined in this way. The pre-injection serum sample from the sow was negative, but positive results were obtained with all the post-injection sera from this animal, and also with the colostrum. All the piglets had positive sera 7 days after birth, but only two out of the six sera that were collected when the piglets were killed were still positive.

The antibody response was less marked in the sow than in the pigs in Table 1, in that none of the post-injection sera from the sow gave more than one precipitation line, and this was reflected in a single, weakened line in the sera of the piglets. The colostrum sample, however, gave two precipitation lines.

No differences were detected between cloned and uncloned, or between particulate and ultrasonically-disintegrated antigens.

Stimulation of antibodies in a pregnant sow with non-formalinized antigen, with subsequent antibody transfer to the litter via the colostrum

In case the formalin treatment in the earlier experiments had reduced the antigenicity of *M. suipneumoniae*, the antigen in this experiment was not formalinized; nor was any adjuvant used. Two intramuscular injections were given 22 and 13 days before the sow farrowed seven live pigs. The antibody titres and the results of challenging the sow and her litter are summarized in Table 3. The sow had IHA and CF serum titres when tested 9 days after the first injection and the colostrum taken at farrowing had high titres by these tests. By 7 days of age, when the litter was first tested, the piglets' sera showed good IHA and CF titres. Compared with the colostrum titres, however, the IHA titres were relatively much lower at this time than the CF titres. The sow and litter were challenged with either high or low doses when the litter was 8 days old and killed 19 or 20 days later.

Apart from piglet 3044, which showed slight collapse histologically, the lung lesions in all these animals were typical of enzootic pneumonia. The mean pneumonic score of the three piglets that were given the high dose of inoculum was nearly 15, and the corresponding score for the four piglets that received the low dose was seven: this difference might have been due to the different levels of challenge. In general, however, the litter was not obviously protected by the colostral antibodies, unless the small lesions in pigs 3049 and 3044 indicated some degree of protection against a low challenge dose. Neither did the injections given to the sow prevent her from developing enzootic pneumonia.

Table 3. *IHA and CF titres developed by a pregnant sow injected with non-formalinized suspension of* M. suipneumoniae, *the titres acquired by the litter from the colostrum, and the effect of challenging the sow and litter with enzootic pneumonia*

	Titres (reciprocals)													
	Serum, 22 days before farrowing*		Serum, 13 days before farrowing†		Colostrum during farrowing		Serum, 7 days after farrowing, 1 day before challenge		Serum, 19 or 20 days after challenge, when killed		Challenge dose		Pneumonic score‡	Mycoplasma score‡
Pig	IHA	CF	IHA	CF	IHA	CF	IHA	CF	IHA	CF	ml.	dilution		
Sow 3051	< 10	< 10	80	40	20,480	320	640	80	640	< 10§	8	1/10	6	2
Piglet 3045	—	—	—	—	—	—	1280	160	320	40	5	1/2·5	8	3
3046	—	—	—	—	—	—	640	320	160	80	4	1/2·5	12	2
3048	—	—	—	—	—	—	1280	80	160	< 10§	4	1/2·5	24	3
3044	—	—	—	—	—	—	1280	320	160	< 10§	4·5	1/250	0·5	0
3047	—	—	—	—	—	—	2560	160	160	< 10§	5·5	1/250	9·5	3
3049	—	—	—	—	—	—	320	40	No serum		5	1/250	1	3
3050	—	—	—	—	—	—	1280	160	80	40	4	1/250	17	4

* Time of first injection
† Time of second injection
‡ The scoring systems are described in Materials and Methods.
§ Partial fixation occurred with these sera to a titre of 1/40 or 1/80.

DISCUSSION

The first six pigs that were challenged after being given formalinized *M. suipneumoniae* antigen plus Freund's complete adjuvant (Table 1) showed no clear resistance to the development of enzootic pneumonia. When a lower challenge dose was used, however, some protection might have occurred (pigs 3031, 3032 and 3030 in Table 2), but only experiments involving larger groups of animals could establish whether this was indeed the case. Nevertheless, it is very likely that our usual challenge inoculum (about 5 ml. of a 1/10 suspension of pneumonic tissue given intranasally) is considerably more potent than the initial challenge that would be experienced by most pigs in the field from airborne infection. The mode of natural infection is also different, in that the farm pig is usually continually exposed to the disease, and the initial, possibly small doses of this type might reinforce an artificially-induced immunity rather than overwhelm it. There is an additional complication affecting these conclusions: farm pigs, even when apparently free from enzootic pneumonia, are more difficult to infect with this disease than hysterectomy-produced pigs (R. F. W. Goodwin and P. Whittlestone, unpublished); this natural resistance, coupled with a specific, artificially-induced resistance, might tip the scales in favour of the pig not developing enzootic pneumonia under all but the heaviest weights of natural infection. Certainly, however, any immunity induced in the present experiments was not comparable with that resulting from the experimentally-induced disease, because the challenge dose employed here was generally similar to the challenge doses used in our previous work, where the pigs recovering from enzootic pneumonia were found to be powerfully immune (Goodwin *et al.* 1969).

One further point should be raised concerning the evaluation of protection. Enzootic pneumonia has a marked debilitating effect in the field but it has frequently been observed that this effect does not often correlate with the extent of the pneumonic lesions at slaughter. One possible explanation for this anomaly is that the causal mycoplasma may exert its influence by inducing some extrapulmonary metabolic derangement. If this were so, the extent of the pneumonic lesions after challenge might not be the best or sole criterion for the effectiveness of artificial immunization—growth rate or conversion ratio should also be taken into account.

Whether any appreciable degree of immunity was induced with killed antigen or not, it seems clear that this immunity did not correlate with either the MI, IHA or CF serum titres; for the higher IHA and CF titres among the challenged pigs in Table 1 appeared to confer no additional benefit, and when pigs 3065 and 3066 (Table 2) became naturally immune they did not develop any higher IHA or CF titres than injected pigs that had not resisted a comparable challenge. Indeed, the MI, IHA and CF titres in these non-immune or poorly immune pigs were generally as high as those in our previous work on the strong immunity following the experimentally-induced disease (Goodwin *et al.* 1969). It seems, therefore, that even if a suitable way of inducing a good immunity by artificial means could be found, the MI, IHA and CF serum titres would not indicate the strength of this immunity.

The immunization of a pregnant sow with non-formalinized antigen did not protect the sow from developing pneumonia; neither was the litter obviously protected by the colostral transfer of antibodies. These results, coupled with the findings in the pigs given formalinized antigen, are not very encouraging as far as the question of a satisfactory vaccine is concerned but they do not, of course, rule out the eventual production of such a vaccine.

In our earlier study of immunity in experimentally-induced enzootic pneumonia (Goodwin *et al.* 1969) all but one of 21 serum samples from 19 pigs that had not been exposed to infection had an IHA titre of less than 1/5; no higher titres, however, were obtained by 22 days after infection, although substantial titres were obtained in all the pigs that had been infected 16 weeks previously. Likewise, in the work now described, the pre-injection titres were all below 1/10 and the IHA titres were slow to develop: four out of the 11 pigs in Table 1 still had titres of less than 1/10, 18 days after their first injection, but substantial IHA titres were recorded by 14 days after the second injection (32 days after the first injection).

The equivalent situation with the CF titres was that, as in the earlier study, the pre-injection titres were all less than 1/10, but the CF titre in the sow had become 1/40, 9 days after the first injection, and by 18 days after their first injection, all but one of the 11 pigs in Table 1 had CF titres of 1/80 or more. The more rapid development of CF titres compared with IHA titres, which occurred in the experimentally-induced disease, was paralleled, therefore, in pigs which had been injected with antigen.

SUMMARY

Hysterectomy-produced, colostrum-deprived pigs were injected twice with formalinized antigen prepared from the J strain of *Mycoplasma suipneumoniae;* the first injection was with Freund's adjuvant and the second injection without adjuvant. The immunity of these animals was tested by inoculating them intranasally with different doses of lung suspension prepared from cases of enzootic pneumonia. Two of the pigs were not killed shortly after infection, but were kept and challenged with enzootic pneumonia in order to compare the serology of experimentally-injected animals with the serology of the immune state following the experimentally-induced disease.

In a second main experiment, a pregnant sow was injected twice with non-formalinized antigen without adjuvant, and her litter was subsequently exposed to the disease at 7 days of age after suckling naturally from birth.

There was no evidence to suggest that the injections had protected the pigs in the first experiment against a high dose of infection, but they may have given some protection against low doses. The piglets suckled by the injected sow were not protected against two different doses of infection.

Serum samples taken at different stages were examined by the metabolic-inhibition (MI) test, the indirect-haemagglutination (IHA) test, the complement-fixation (CF) test and the gel-diffusion precipitin test, using *M. suipneumoniae* as antigen.

Serum samples taken before injection in the first experiment were all negative

in the MI test and they became positive after the injections of antigen. However, the highest MI titres obtained were not associated with obvious immunity; nor was the development of true immunity after experimental infection associated with a change in MI titre.

In the first experiment, substantial IHA titres (over 20,000) were recorded by 14 days after the second injection of antigen. Again, there was no correlation between the IHA titres and the area of pneumonia following experimental infection. In the sow experiment, IHA titres developed after the first injection and increased after the second; a high IHA titre occurred in the colostrum and titres of 320 or more were present in the piglets 7 days after birth.

The CF titres appeared earlier than the IHA titres but did not increase so markedly thereafter. Once more, there was no correlation between the titre before infection and the area of pneumonia afterwards.

In the gel-diffusion test, precipitins were demonstrated in all the post-injection serum samples tested, most of the samples being positive after the first injection. Precipitins were also demonstrated in the colostrum of the injected sow and in her uninjected litter at 7 days of age.

From these experiments it was concluded that, as judged by the development of pneumonic lesions and in marked contrast to the known immunizing effect of the disease itself, injections of antigen given in this manner had little or no protective effect against the dose levels of infection used. Nevertheless, the titres obtained in the MI, IHA and CF tests were comparable with those obtained earlier in pigs that were strongly immune, which provides further evidence for the suggestion that these tests do not measure protective immunity.

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Terminal bronchopneumonia. A bacteriological and histological study of 111 necropsies

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Terminal bronchopneumonia usually occurs in patients already debilitated by serious illness. It may be caused by highly virulent bacteria or by opportunistic organisms of low pathogenicity and cases occurring in hospital may be a sensitive indicator of the pathogens present in the hospital environment. In the past 25 years great changes have occurred in the pattern of hospital infection, attributable largely to the introduction of antibiotics. There has been a decline in importance of many familiar pathogens and *Staphylococcus aureus* has become a major problem. More recently there have been indications that the Gram-negative bacilli are becoming more important causes of infection (Finland, Jones & Barnes, 1959; Kneeland & Price, 1960; Lepper, 1963). Some of these bacteria are of low grade pathogenicity but they can produce severe infections in patients with diminished resistance.

This investigation was an attempt to determine the pattern of infection in terminal bronchopneumonia in a series of necropsies.

MATERIALS AND METHODS

A total of 111 consecutive necropsies in seven mental hospitals, during the months of February to October 1967, were investigated histologically and bacteriologically. The average age of the patients was about 70 years (range 20–92) and the sex distribution was approximately equal. When necropsy was not possible within a few hours of death, the body was refrigerated. All lungs were examined histologically and other organs as indicated by macroscopic appearances.

Bacterial isolation and identification

Swabs for bacteriological investigation were taken from lungs, spleen and colon. The body was opened without disturbing the viscera, a new pair of disposable rubber gloves being worn for each necropsy. With a hand inserted in the pleural cavity the posterior surface of the lung was palpated from apex to base; without withdrawing the hand the lung was lifted enough to permit swabs to be plunged into lung tissue through the untouched anterolateral surface selecting consolidated areas when present. Similarly the spleen was lifted by its pedicle and swabs were pushed through the untouched anterior surface. Faeces were sampled by pushing a swab through an untouched surface of the descending colon.

Swabs from the lung and spleen were plated on blood agar, chocolate agar, milk salt agar (Cruickshank, 1965) and MacConkey's agar; swabs from the colon were plated on MacConkey's agar only. Plates were incubated aerobically at 37°C. for 48 hr.; blood agar plates were duplicated for anaerobic culture. The approximate numbers of colonies on primary plates were counted and the organisms identified as fully as possible using the methods of Cowan & Steel (1965). Strains of *Staph. aureus* were phage typed and tested for sensitivity to penicillin and tetracycline and Gram-negative bacilli to sulphonamides, tetracycline, streptomycin and chloramphenicol, using lysed blood agar and 'Oxoid' sensitivity disks. The Oxford staphylococcus and a sensitive strain of *Escherichia coli* were included in each batch of tests as controls.

Serological typing of E. coli.

A very limited range of antisera was available; 01, 04, 06, 018*ab*, 018*ac*, 065, 069 and 075 were kindly supplied by Dr A. R. Foder of The Communicable Disease Centre, Atlanta, U.S.A.; 025 and 050 were prepared locally. The agglutination titres of these sera ranged from 1/2000 to 1/10,000.

For serotype determination, single colonies from primary cultures were sub-cultured on nutrient agar and, after biochemical confirmation of identity, saline suspensions were prepared, washed, boiled for 1 hr. and diluted to a final concentration of 250×10^6 organisms per ml. Equal quantities of suspension and antiserum diluted to one half titre were incubated at 50°C. in round-bottom tubes for 20 hr., when the presence of agglutination was read macroscopically. Five separate colonies of strains isolated from faeces were tested and two colonies of strains from other sites.

RESULTS

In almost all of the 111 necropsies a serious underlying disease was present—mainly carcinoma or cardiovascular disease. Sixty-five patients had histological evidence of acute inflammation in the lungs, and of these 53 had pneumonia and 12 bronchitis, bronchiolitis or lung abscess. The remaining 46 patients had no histological evidence of pulmonary inflammation and were used as a control group.

Table 1 shows the relation between the presence of inflammatory lesions in the lung and the bacterial flora of the lung and spleen. It was expected that contaminant bacteria would occasionally be isolated from normal lung but that at least moderate numbers would be present in inflamed lung tissue; growths of less than 20 colonies per plate were therefore ignored. In fact it was found that most lung cultures were either sterile or exceeded 20 colonies per plate. The spleen on the other hand seemed less accessible to contamination and any bacterial growth was recorded.

Both *E. coli* and *Staph. aureus* were isolated significantly more frequently from inflamed lungs than from the lungs of controls ($P < 0{\cdot}0001$ and $P = 0{\cdot}0469$ respectively). *Haemophilus influenzae*, *Proteus mirabilis* and *Streptococcus pneumoniae* isolations had the same distribution but numbers were not significant. No anaerobic organisms were isolated.

Swabs from the spleen were cultured in the last 92 necropsies of the series, and of 53 patients with inflammatory lesions in the lung an organism was grown from the spleen in 25 whereas only eight of 39 controls were positive. Again, *E. coli* ($P = 0{\cdot}0267$) and *Staph. aureus* ($P = 0{\cdot}0050$) were significantly more frequent in patients

Table 1. *Bacteria isolated from the lungs and spleen from* 111 *necropsies*

	Lung. Pulmonary inflammation		Spleen. Pulmonary inflammation	
Bacteria isolated*	Present, 65 patients	Absent, 46 patients	Present, 53 patients	Absent, 39 patients
Staph. aureus	20 (31)	7 (15)	12 (23)	1 (3)
E. coli	22 (34)	2 (4)	9 (17)	1 (3)
P. mirabilis	7 (11)	3 (7)	5 (9)	1 (3)
Other coliform organisms†	4 (6)	3 (7)	3 (6)	1 (3)
Str. pneumoniae	2 (3)	0	0	1 (3)
β-haemolytic streptococci (groups B, C or G)	2 (3)	2 (4)	1 (2)	0
Str. faecalis	3 (5)	2 (4)	3 (6)	0
Str. viridans	2 (3)	5 (11)	1 (2)	2 (5)
H. influenzae	5 (8)	0	0	0
Past. haemolytica var. *ureae*	1 (2)	0	0	0
Staph. saprophyticus	2 (3)	2 (4)	1 (2)	3 (8)
No significant growth‡ or no growth	20 (31)	27 (59)	28 (53)	31 (80)

The figures in parentheses are percentages in each group.
* A number of bacteria were present in mixed culture.
† Includes *Enterobacter aerogenes. Ent. cloacae, Klebsiella aerogenes* and Providence.
‡ See text.

Table 2. *Effect of time interval between death and necropsy on bacterial isolations*

Pneumonic lesions	Site	Time between death and necropsy (hr.)	Number of necropsies	Number of patients yielding *Staph. aureus*	Entero-bacteria	No signi-ficant growth
Absent	Lung	< 24	11	1 (9)	2 (18)	7 (64)
		> 24*	35	6 (17)	5 (14)	20 (57)
	Spleen	< 24	10	0	0	9 (90)
		> 24†	29	1 (3)	3 (10)	22 (80)
Present	Lung	< 24	12	3 (25)	8 (67)	4 (33)
		> 24‡	53	17 (32)	22 (42)	16 (30)
	Spleen	< 24	11	2 (18)	3 (27)	5 (45)
		> 24§	42	10 (24)	13 (31)	23 (55)

Figures in parentheses indicate percentages in each group.
* Mean 2·1 days. Range 1–6 days.
† Mean 2·2 days. Range 1–6 days.
‡ Mean 1·7 days. Range 1–5 days.
§ Mean 1·8 days. Range 1–5 days.

with pulmonary inflammation than in controls. Apart from the *Staph. saprophyticus* and *Str. viridans* other organisms showed the same distribution but differences were not significant.

Bacteria isolated from the spleen were usually also present in significant numbers in inflamed lungs. In fact 75 % of bacterial isolations from the spleen were accompanied by the isolation of an apparently identical species from the lung. Moreover, in 10 cases of *Staph. aureus* infection, the same phage type was present in both sites in eight cases; and in nine infections with typable *E. coli* the same serotype was isolated from both sites in eight.

The probabilities quoted above were calculated by Fisher's Exact Test (Fisher, 1950), using one tail only of the distribution. The isolation rates of *E. coli* from the lungs and of *Staph. aureus* from the spleen were significantly higher in the group with pulmonary inflammation by a one- or two-sided test. In view of the good correlation between isolations from the spleen and inflamed lung, the possibility of differences for these bacteria occurring in opposite directions in these two sites could be discounted. A single-sided test was therefore used for these particular probability estimates, but all subsequent tests were two-sided.

Table 3. *Effect of antibiotic therapy on pulmonary flora*

Inflammatory lesions in lungs	Antibiotics given	Total no. of patients	No. of patients with bacteria in lungs			
			E. coli	*Staph aureus*	Other* organisms	All* organisms
Present	Yes	34	7 (21)	10 (29)	11 (32)	21 (62)
	No	31	15 (48)	10 (32)	13 (42)	24 (77)
Absent	Yes	13	1 (8)	2 (15)	1 (8)	4 (31)
	No	33	1 (3)	5 (15)	3 (9)	11 (13)

Total necropsies, 111.
Numbers in parentheses indicate percentages of group total.
* *Str. viridans* and *Staph. saprophyticus* were considered to be of no significance and were excluded.

Necropsies were usually conducted within 2 days of death; 59 % were completed by the day after death and only 16 % were delayed for more than 3 days. There was no significant difference between isolation rates from necropsies conducted within 24 hr. of death and those in which there was delay (Table 2). The possibility that residual antibiotics in the tissues had prevented post mortem bacterial multiplication was considered, but it was found that similar proportions of all groups had received antibiotics.

Table 3 shows the effects of antibiotics on the pulmonary flora. *E. coli* was isolated more frequently from the patients with pulmonary inflammation who were not receiving antibiotics than those who were ($P = 0{\cdot}021$). It appeared therefore that antibiotics had probably suppressed the growth of *E. coli* in the treated group and that the isolation rate of 48 % in the untreated group was a better estimate of the incidence of *E. coli* infection.

The most commonly used antibiotic was tetracycline and the reduced *E. coli*

isolation rate in treated patients suggested that most strains were sensitive. Of the 15 strains of *E. coli* isolated from patients with pulmonary inflammation 12 were sensitive.

Antibiotic therapy made little difference to the isolation rates of *Staph. aureus* from the lungs. As none of the strains isolated from treated patients was sensitive to the antibiotic given, this was not surprising. Of 20 strains of *Staph. aureus* isolated from inflamed lungs 13 were tetracycline resistant and of these nine belonged to types which have often been associated with outbreaks of sepsis in surgical wards (eight 52/52A/80/81, 80/81 and similar patterns; one 84/85).

Using a very limited range of antisera, an attempt was made to determine the serotype of strains of *E. coli* isolated from inflammatory lesions of the lung and various other extra-intestinal sites in 25 patients (lung and spleen, eight patients; lung only, 13; spleen only, two; lung and rib abscess, one; liver abscess, one). Two colonies were tested from each site and in only two patients was a mixture of types demonstrated. Fourteen of 25 patients with *E. coli*-infected lesions had a typable strain in their lesion (06, 018, 050 or 075). The isolation rate from the faeces of these patients was similar; six of 12 tested carried one or other of these four strains in their faeces. On the other hand only one of 19 patients who did not have an *E. coli* infected lesion was carrying one of these strains (06, 018, 050 or 075) in their faeces.

An extrapulmonary lesion which could have been the source of Gram-negative metastatic bacterial infection was sought in the 53 patients with pneumonia. (Table 4.) Eight such lesions were found in 24 patients with enterobacterial pneumonia, whereas only one lesion ($P = 0{\cdot}0074$) was found in 29 patients with pneumonia associated with other bacteria.

Table 4. *Extrapulmonary lesions which could have been a source of enterobacteria*

(53 necropsies on patients with pneumonia)

Lesion	Isolation of enterobacteria from lung	
	Present	Absent
Perforated gastric carcinoma	1	—
Cholecystitis with liver abscesses	1	—
Diverticulitis of sigmoid colon	1	—
Pelvic abscess	1	—
Abscess associated with carcinoma of the colon	1	—
Necrotic strangulated bowel	1	—
Mesenteric thrombosis	2	—
Liver abscess	—	1
None	16	28
Total	24	29

DISCUSSION

For many years post mortem bacteriological investigations were thought to be of little value, as it was generally believed that the tissues were flooded with

bacteria, either before or after death. More recent work suggests that after the first few hours there is insignificant post mortem bacterial multiplication, and that whatever change in distribution of bacteria takes place at about the time of death its extent is not sufficient to obscure the true bacteriological picture (Burn, 1934; Smillie & Duerschner, 1947*a*; Kurtin, 1958; Kneeland & Price, 1960). Our results support this view; bacterial isolation rates from lungs and spleens changed insignificantly when necropsy was delayed and the correlation between histological and bacteriological results is incompatible with the possibility of the latter arising from random flooding with bacteria or from chance contamination due to the technique of swab collection.

It is difficult to assess the relative importance of different bacteria in the 65 patients with pulmonary inflammation because in 20 the bacterial growth was not significant, and in 21 it was mixed. However, *E. coli* was isolated most frequently and as antibiotics had reduced its isolation rate it would appear that nearly half the pneumonias were due to this organism.

Staph. aureus was isolated significantly more frequently from inflamed lungs than from the lungs of controls showing no inflammation. Although the controls had a high isolation rate, most were elderly patients with a serious underlying disease and 70% had histological evidence of pulmonary oedema which was perhaps premonitory of inflammatory change. A P.H.L.S. necropsy survey (Report, 1966) found that of 125 patients who died outside hospital only six (4·8%) had *Staph. aureus* in the lungs. It seems, therefore, that *Staph. aureus* might have been a contributory cause of death in something like 15% of patients with pneumonia.

Smillie & Duerschner (1947*b*) found that *Str. pyogenes*, *Str. pneumoniae*, and *H. influenzae* were the most common pathogens in terminal bronchopneumonia. *Staph. aureus* was frequently found, but it was also commonly present in the lungs of patients who did not have pneumonia; the authors concluded that it was sometimes a cause but less frequently than other organisms. These results were probably representative of the pre-antibiotic era, but subsequent reports indicated a changing pattern. Kneeland & Price (1960), and Lepper (1963), found that streptococci, pneumococci and *H. influenzae* had almost disappeared as causes of bronchopneumonia, their place being taken by *Staph. aureus* and to a lesser extent by Gram-negative bacilli. Our investigation also shows this pattern, and further suggests that *E. coli* may at times exceed *Staph. aureus* in frequency as a cause of terminal bronchopneumonia.

A question posed by this change in pattern is why has it occurred? Smillie & Duerschner (1947*a*, *b*) found that though organisms present in the nasopharynx could also be recovered from the trachea, they were not normally found beyond its bifurcation, but in terminal bronchopneumonia the more invasive of the nasopharyngeal bacteria passed this barrier to invade the lungs of patients whose resistance was impaired. Whether *Str. pyogenes*, *Str. pneumoniae* and *H. influenzae* have declined in virulence is uncertain, but perhaps a more important question is why they are being replaced by *Staph. aureus* and Gram-negative bacilli. Are they invading tissues from which they were previously excluded by more virulent bacteria? Or are the current hospital strains more virulent or prevalent than hitherto?

In the case of *Staph. aureus*, its increasing prevalence in terminal bronchopneumonia follows the recent trend of staphylococcal sepsis in general hospitals and is probably due to much the same factors. The most important of these is the high carriage rate among hospital patients and staff of virulent 'epidemic' types of staphyloccoci, the selection of which has been favoured by the use of antibiotics. Although our patients were in mental hospitals, the predominance of tetracycline-resistant strains and 'epidemic' phage types suggests that the staphylococcal environment was similar to that of general hospitals.

The large number of *E. coli* infections of the lung in our series was the most striking feature but the reason for it is not clear. A number of investigations have shown that only a few serological types of *E. coli* are responsible for most coliform infections (e.g. Turck, Petersdorf & Fournier, 1962; Kennedy, Plorde & Petersdorf, 1965). Much of this work has concentrated on urinary infection but our investigation suggests that in terminal bronchopneumonia there may be a high proportion of infections by certain serotypes (06, 075 and 018). Kennedy *et al.* (1965) and Winterbauer, Turck & Petersdorf (1967) found that types 04, 06 and 075 occur more frequently in the faeces of patients and staff in hospital and the carrier rate among patients was directly related to the time spent in hospital; inanimate objects in the wards were rarely contaminated and they therefore suggested that antibiotic treatment or other factors present in hospital patients might encourage spread from the endogenous bowel flora. Since, however, 40% of the hospital staff in their study were carrying these serotypes there would seem to have been ample opportunity for transmission by direct personal contact.

Whatever their source might be these serotypes readily colonise the faeces of hospital patients, but how do they reach the lungs? *E. coli* does not normally colonize the nasopharynx although it sometimes does so in infants. Recently, however, Stratford, Gallus, Matthiesson & Dixson (1968), reported that in severely ill patients the predominant bacterial flora of the skin, nose and throat changed from Gram-positive cocci to Gram-negative bacilli—mainly *E. coli* and *Proteus* spp. —and this change was not related to antibiotic therapy. If this occurs very commonly, invasion of the lungs via the air passages would be a likely route. Alternatively, bacteraemic spread from the gut or other extra-pulmonary inflammatory lesion could occur. Tillotson & Lerner (1967) reported 20 cases of pneumonia due to *E. coli* in which the bacilli were thought to reach the lungs via bacteraemias from sources in the kidneys or gastro-intestinal tract. In our series only eight of 22 patients with enterobacterial pneumonia were shown to have such a lesion, but it seems likely that this is an important route of infection (Table 4).

Whether the selection of these serotypes of *E. coli* is favoured by the use of antibiotics is uncertain. Since the strains isolated in this survey showed no increase in antibiotic resistance, we have no evidence of it, although Winterbauer *et al.* (1967) found that patients receiving broad-spectrum antibiotics had a tendency to acquire *E. coli* of O groups 4, 6 and 75 and felt that this was the result of alteration of the intestinal flora by antibiotics.

SUMMARY

One hundred and eleven consecutive deaths in seven mental hospitals during the months of February to October 1967 were investigated histologically and bacteriologically.

Bacteria were present in the lung tissue significantly more frequently when inflammation was present than when it was absent and the differences were significant for both *Escherichia coli* and *Staphylococcus aureus*; *E. coli* was isolated from 22 (34 %) of the 65 patients with inflamed lungs compared with two (4 %) of 46 control patients and the corresponding figures for *Staph. aureus* were 20 (31 %) of 65 patients compared with seven (15 %) of 46 control patients.

Some strains of *E. coli* were serotyped using antisera against O antigens 1, 4, 6, 18*ab*, 18*ac*, 25, 50, 65, 69 and 75 and the strains most commonly found in the inflamed lungs, spleens and other inflammatory lesions of 25 patients were 06, 018 and 075. These strains were isolated more frequently from these sites than from the faeces of a group of 19 patients with no such lesions.

In this series *E. coli* was the commonest organism to be associated with terminal bronchopneumonia and the possible reasons for this are discussed.

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Antibody response and reactions to aqueous influenza vaccine, simple emulsion vaccine and multiple emulsion vaccine. A report to the Medical Research Council Committee on influenza and other respiratory virus vaccines*

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Influenza vaccines prepared with a mineral oil adjuvant induce a substantial and durable antibody response. However, vaccines containing mineral oil in simple emulsion (water-in-oil emulsion) sometimes produce persistent local reactions (Medical Research Council, 1964). The development of a redispersed 'multiple' emulsion in which antigen is incorporated as an oil-in-water emulsion starting from an original water-in-oil emulsion was described by Herbert (1965). Such multiple emulsion vaccine has a lesser viscosity than simple emulsions and might therefore produce a substantial antibody response with fewer reactions. In this investigation a comparison has been made of the antibody response and short-term vaccination reactions after aqueous influenza vaccine, influenza vaccine containing a mineral oil in simple emulsion, and influenza vaccine containing mineral oil in multiple emulsion.

GENERAL PLAN

The investigation began in November 1966. There were approximately 300 participants all of whom were volunteers employed at the oil refinery at Shell Haven, Essex. They were aged 20–59 years. A blood sample was withdrawn from each volunteer and, immediately after, each received an intramuscular injection of one of the following four vaccines: either aqueous vaccine, or adjuvant simple emulsion vaccine, or adjuvant multiple emulsion vaccine, or, as a control, rhinovirus

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vaccine. The three influenza vaccines each contained identical virus strains from the same virus pool. The four vaccines were given in strict rotation in the order in which each volunteer arrived at the clinic.

Twenty-four hours after vaccination as many as possible of the participants were seen and asked about the occurrence of systemic reactions and the vaccination sites were examined. Thereafter, each participant was invited to complete and return a questionnaire stating whether or not any reactions occurred 72 hr. after vaccination and giving appropriate descriptions; those who failed to return the questionnaire were followed up and examined. Further blood samples were withdrawn approximately 3 and 12 months after vaccination. The haemagglutination-inhibiting (HI) antibody titres in the pre-vaccination serum and the two post-vaccination sera were titrated in parallel. During the 3 months after vaccination an account was kept of the sickness absence of the volunteers.

METHODS

The vaccines

These were prepared by Evans Medical Ltd.

Aqueous vaccine. The aqueous vaccine was a saline suspension of the following composition (given in haemagglutinating units (Salk)): A_2/England/12/64, 5250; A_2/England/76/66, 2500; B/England/939/59, 3250; B/England/5/66, 3250. The material was inactivated by the use of formalin (1/4000) followed by betapropiolactone (1/1000), concentrated by differential centrifugation and, after appropriate dilution in phosphate buffered saline containing 0·013% thiomersal, was distributed into 1 ml. ampoules.

Simple emulsion. Using the same strains, a 50/50 water-in-oil emulsion was prepared with Drakeol 6 VR and Arlacel A. (The constituents of the emulsion and the final vaccine passed the tests described by Berlin (1962).) The emulsion was then dispensed into disposable syringes so that a dose of 0·25 ml. contained one quarter of the haemagglutinin content of the simple aqueous vaccine for each strain.

Multiple emulsion. This was prepared by the addition of one-half volume of phosphate saline containing Tween 80 to 1 volume of simple water in oil/oil in water emulsion. The mixture was then re-emulsified to produce a double emulsion. After emulsification the material was dispensed into disposable syringes in 0·375 ml. amounts to give the same HA content as that of the simple emulsion.

All vaccines were tested for conformity with the Therapeutic Substances Act requirements.

Antibody titrations

The serological tests were carried out in perspex trays by the method recommended by the World Health Organization Expert Committee on Influenza (1953) using a final dilution of four agglutinating doses of antigen. Readings were made after 60 min. at room temperature, the end-point being taken as 50% inhibition of agglutination or calculated by interpolation when the 50% end-point fell between two dilutions.

All sera were treated with *Vibrio cholerae* enzyme (R.D.E.) and pre- and post-vaccination sera of each individual were always included in the same test.

RESULTS

Vaccination reactions and sickness absence

A total of 302 volunteers were vaccinated. Their ages ranged from 20 to 59 years and all but 18 were males.

Of these participants only 162 had all the necessary serum samples withdrawn. Of these so tested 41 received aqueous vaccine; 39 simple emulsion vaccine; 42 multiple emulsion vaccine; and 40 rhinovirus (control) vaccine.

Table 1. *Local and general reactions up to 72 hr after vaccination, according to group*

Type of reaction	Nos. showing reactions after			
	Aqueous	Simple emulsion	Multiple emulsion	Rhinovirus (control)
Local	6 (8)	6 (8)	20 (26)	0
General	21 (27)	27 (36)	29 (38)	10 (14)
No reaction	51 (65)	41 (55)	27 (36)	64 (86)
Total	78	74	76	74

Figures in parentheses indicate percentages.

There were no severe local reactions, but mild erythema, swelling and discomfort were common; none of the reactions was troublesome. The frequency of local reactions after multiple emulsion vaccine was much greater than with the other vaccines, but this increase was noted 24 hr. after vaccination only and was a reflexion of the large number of complaints of only slight discomfort from the recipients of this vaccine. Mild general reactions consisting of headache, muscular pain and malaise were common complaints after all the influenza vaccines and were less frequent after rhinovirus vaccine (Table 1).

Ninety-one participants had been immunized about 2 years previously with an influenza vaccine containing an oil adjuvant; the reactions in these participants were not more frequent or severe than those among persons who had not been immunized previously.

During the 4 months after immunization there was no outbreak of influenza and, as might be expected, the time lost by sickness absence from all causes was the same in each of the vaccination groups. This similarity was maintained for the 12 months after inoculation. However, irrespective of the vaccine given the sickness time lost by absence from all causes was greater among participants with reactions after vaccination than among those with no reactions. Thus of the 119 participants with a reaction, either local or general, 48 (40 %) had at least one spell of sickness during the 3 months after vaccination, whereas of the 183 persons without reactions, 48 (26 %) had at least one spell of sickness. These differences attain statistical significance at the 5 % level.

Antibody titres before vaccination

Each of the vaccine groups contained participants with and without detectable antibody (Table 2).

Fifty-three participants from whom serum samples were obtained had been vaccinated 2 years previously with oil adjuvant vaccine containing 3500 HI units of the following four virus strains: A/Singapore/1/57, B/England/939/59, A/England/1/61 and B/Taiwan/4/62.

Of the 53 participants who had been previously vaccinated nearly 91 % had antibody to the A2 virus and 66 % antibody to the B virus (Table 3) whereas only about 40 % of persons who were not vaccinated had antibody to A2 virus and 23 % antibody to the B virus.

Table 2. *Antibody titres before vaccination according to group*

		Antibody titres							
		< 12		12–48		> 48		Geometric mean titres	
Group	No.	A2	B	A2	B	A2	B	A2	B
Aqueous	41	20	27	15	11	6	3	16	10
Simple emulsion	39	17	24	12	12	10	3	20	12
Multiple emulsion	42	18	27	19	13	5	2	17	10
Rhinovirus (control)	40	16	24	12	12	12	4	25	13

Table 3. *Antibody titres in first serum sample according to history of previous influenza vaccination*

			Antibody titres					
	Total no.		< 12		12–48		> 48	
Group	A2	B	A2	B	A2	B	A2	B
Previously vaccinated	53	53	5 (9)	18 (34)	22 (42)	24 (45)	26 (49)	11(21)
Not previously vaccinated	109	109	66 (61)	84 (77)	36 (33)	24 (22)	7 (6)	1 (1)

Figures in parentheses indicate percentages.

Antibody response to vaccination

The geometric means of the antibody titres before vaccination and at approximately 3 and 12 months after vaccination are shown in Table 4. It is evident that there was a very substantial increase in antibody to the A2 component after each of the three influenza vaccines, but that this increase was not the same after each vaccine. Three months after aqueous vaccine the mean titre was approximately 11 times greater than the prevaccination sample; after simple emulsion vaccine 16 times greater; and after multiple emulsion vaccine 27 times greater. One year after vaccination the titres produced by each vaccine had declined substantially to roughly half the titres observed at 3 months. However, of those without antibody

before vaccination and who thus might be expected to be specially susceptible to a natural infection with influenza virus, 18 of 20 given aqueous vaccine possessed antibody to the A 2 virus at the end of 1 year. All 17 participants without initial antibody who were given simple emulsion vaccine and all 18 participants without initial antibody given multiple emulsion vaccine had antibody at 1 year.

Table 4. *Geometric mean of antibody titres in 1st, 2nd and 3rd serum samples, i.e. immediately before vaccination, approximately three months after vaccination and approximately 12 months after vaccination*

		Geometric mean titre					
		A 2			B		
Vaccine	No.	1st	2nd	3rd	1st	2nd	3rd
Aqueous	41	16	171	102	10	34	23
Simple emulsion	39	20	334	166	12	89	52
Multiple emulsion	42	17	461	206	10	132	70
Rhinovirus (control)	40	25	26	25	13	13	12

The mean antibody titre to the influenza B virus component showed a much smaller increase at 3 months than the increase to the A 2 virus component, but the increase was less after aqueous vaccine (approximately three-fold) than to the simple emulsion and multiple emulsion vaccines (seven-fold and thirteen-fold respectively). With each vaccine the mean titre declined 1 year after vaccination. For those without initial influenza B antibody titres 15 of 27 given aqueous vaccine, 22 of 24 given simple emulsion vaccine and 25 of 27 given multiple emulsion vaccine had antibody at 12 months. The antibody response in the recipients of the influenza vaccine was not due to natural infection since the geometric mean titres of the control group showed little alteration throughout.

COMMENT

In this investigation the influenza vaccine under test contained a mineral oil adjuvant, Drakeol 6 VR and Arlacel A, prepared as a multiple emulsion. This preparation is less viscid than a simple emulsion preparation and might thus be less likely to produce local reactions; on the other hand it might also produce a less intense or durable antigenic stimulus. The multiple emulsion preparation was therefore compared with a vaccine containing the same adjuvants prepared in simple emulsion, and with an aqueous vaccine.

Although reactions to the multiple emulsion vaccine were more frequent than to the aqueous and simple emulsion preparations, the reactions were slight and in themselves would not constitute a serious drawback to the more extensive use of the vaccine. The observation that sickness absence during the subsequent follow-up was greater among participants who complained about reactions than among those who did not, may reflect the fact that some individuals are constitutionally more inclined to complain of minor symptoms than others, whether these symptoms are the result of vaccination or of natural ills.

The antibody response induced by the multiple emulsion preparation was very satisfactory, greater than the response to the simple emulsion vaccine and substantially greater than to the aqueous vaccine. The reversion of the emulsion to an oil-in-water suspension did not, therefore, impair its adjuvant properties.

With each vaccine the antibody titres observed soon after vaccination declined substantially by 12 months. Despite this decline and irrespective of the vaccine given, almost all the participants who had no antibody to the A2 virus before vaccination had antibody at 12 months. Almost all the participants without initial antibody to the influenza B virus and who received either simple or multiple emulsion vaccine had antibody at 12 months, but this was not so for the corresponding participants given aqueous vaccine.

The findings suggest that the multiple emulsion vaccine is at least as effective as the standard oil-emulsion preparation, but provide no information about the frequency and character of late reactions or of the value of the vaccine for routine use. A prolonged and comprehensive examination of this aspect would be required before its routine use could be considered.

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Streptococcal infection in young pigs

III. The immunity of adult pigs investigated by the bactericidal test

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INTRODUCTION

Outbreaks of streptococcal infection in young pigs have been described in England by Field, Buntain & Done (1954) and in the Netherlands by de Moor (1963). The causative organisms are capsulated, haemolytic cocci of specific serological type belonging to *Streptococcus suis*, a subgroup of group D (Elliott, 1966). Piglets susceptible to infection are generally less than 6 weeks of age. In such animals the streptococci gain entrance probably through the respiratory tract, whence they invade the bloodstream, producing a sustained bacteriaemia and infections of the joints and meninges. Some animals die within a few days of infection, others recover completely. Elliott, Alexander & Thomas (1966) showed that the condition can be produced experimentally by spraying broth cultures of *S. suis* into the upper respiratory tract of susceptible piglets but adult animals are normally immune both to the naturally occurring and to the experimentally produced disease.

An analogous condition, joint ill, occurs in lambs and is often caused by a streptococcus of specific type belonging to group C (Blakemore, Elliott & Hart-Mercer, 1941). The resistance of adult sheep to this infection is reflected by the ability of their blood to inhibit the multiplication of joint-ill streptococci *in vitro*; no such inhibition occurs in the blood of susceptible lambs. The present investigation was undertaken to determine whether a similar relationship between susceptibility to infection and bacteriostatic power of the blood exists in *S. suis* infection of pigs, and if so, to characterize the responsible component in the blood.

MATERIALS AND METHODS

The bactericidal test

A modification of the method described by Lancefield (1957) was used throughout this investigation.

Pig blood and serum

Large white and Landrace breed pigs up to 2 years of age were used as the source of blood or serum. One or other of the following methods was used in rearing

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the pigs: (1) reared under conventional conditions; (2) weaned from 2 to 5 days after birth and reared on milk substitute (Amvilac No. 1*); (3) hysterectomy delivered, colostrum-deprived pigs reared under hygienic conditions in isolation. Such gnotobiotic pigs used as a source of serum in some experiments were reared either at the Royal Veterinary College, London, or at Ontario Veterinary College, Guelph, (Yorkshire breed pigs).

Venepuncture

The blood for test was taken from the anterior jugular vein without anaesthetic and delivered into tubes containing heparin (Pularin) 1000 units in 0·2 ml. saline.

Rotation of inoculated blood

After inoculation with streptococci the blood was distributed in 0·4 ml. amounts into glass vials 35 mm × 12 mm. sealed by a screw-on, metal cap lined with a silicone rubber cushion. The vials were placed in a mixing machine in which they rotated end over end at six rev./min. at 37°C.

Streptococcal cultures

Four identical, capsulated strains designed PM1, PM23A, PM32, and C22N were used throughout. A non-capsulated strain, designated A227 and thought to be derived from PM1, was also used. All these strains have been previously described (Elliott, 1966). For use in the bactericidal test the streptococci were grown in 5% horse blood broth for 16 hours at 37°C, and the culture then diluted appropriately in nutrient broth before inoculating into the pig blood.

Streptococcal counts

Two methods were used to enumerate colony forming units (C.F.U.). In early experiments a platinum loop, 3 mm. in diameter, was used to transfer blood samples to the surface of horse blood agar on which they were spread and incubated at 37°C. for 18 hr. before counting the colonies that developed. In later experiments, colony forming units were counted in pour-plates incorporating 0·1 ml. blood samples in 5% horse blood agar; these were incubated at 37°C. for 18 hr. This method was used for counts of broth cultures throughout.

All counts were made in duplicate and expressed as the mean of the C.F.U.

Serum fractionation

Ion exchange chromatography. DEAE cellulose (D.E. 32, Whatman), 800 ml., was packed in a column 3 cm. × 90 cm. The sample was applied in 0·02 M phosphate buffer, pH 7·0, containing 0·01 M sodium azide, and an exclusion peak was collected. Because of difficulty in testing a large number of fractions for bactericidal activity, elution was carried out step-wise using increasing concentrations of NaCl in the starting buffer. The eluate from the column was passed through a Uvicord analyser. The protein-containing material from each elution step was pooled and precipitated

* Amvilac is an antibiotic-free milk substitute, marketed by Glaxo Laboratories, Greenford, Middlesex.

by half saturation with ammonium sulphate. The precipitate was harvested, redissolved in a minimum quantity of water and dialysed against physiological saline.

Gel filtration. This was performed on G.200 Sephadex, 2 l., in a column 5 cm. × 100 cm. (Pharmacia) using 0·1 M Tris buffer, pH 7·2, containing 0·5 M-NaCl and 0·015 M sodium azide. Blue dextran 2000 and haemoglobin were used as markers. The column effluent was passed through the Uvicord analyser. Pooled fractions were precipitated by half saturation with ammonium sulphate, the precipitate dissolved in a minimum quantity of water and dialysed against physiological saline.

All fractions were sterilized by filtration before testing.

Mercaptoethanol treatment. Samples were treated with 0·1 M 2-mercaptoethanol for 2 hr. at 37°C. The mercaptoethanol was then removed by dialysis against several changes of saline.

Protein estimations were made by Folin's method and read against a standard curve prepared with human IgG.

EXPERIMENTAL

Effect of capsules on survival of streptococci in porcine blood

Streptococci isolated from the naturally occurring disease are capsulated. Our first experiments, therefore, sought to determine whether the capsule influenced the survival of the cocci in normal piglet blood.

Table 1. *Effect of capsulation on survival of* Streptococcus suis *in blood of piglet no.* 2

S. suis added to piglet blood (0·4 ml.)		C.F.U. in loopfuls from piglet blood after rotation at 37°C, for stated period		
Designation	C.F.U. in inoculum (0·1 ml.)	0 hr.	3 hr.	6 hr.
Strain A 227 Noncapsulated	192	2	0	0
Strain PM 1 Capsulated	256	2	+ +	+ +

In this and succeeding tables C.F.U. signifies colony forming units; + + signifies innumerable colony forming units.

Two samples of heparinized blood from a 16-day-old piglet (No. 2) were inoculated with 0·1 ml. of a suitably diluted, 16 hr. culture of *S. suis*; one sample was inoculated with a capsulated strain (Strain PM 1), the other with the noncapsulated variant (Strain A 227). The inoculated blood samples were distributed in 0·4 ml. amounts in vials and these were rotated at 37°C. Duplicate loopfuls of blood were taken from duplicate vials before and after rotation for 3 and 6 hr. and the colony forming units (C.F.U.) counted as described under Methods.

The results of this experiment are shown in Table 1. It can be seen that whereas the capsulated cocci grew freely under these conditions, the noncapsulated variant failed to do so. Thirty-five samples of blood from 17 piglets, conventionally reared and less than 6 weeks of age, were tested against the noncapsulated strain A 227.

In none did the cocci show any multiplication. Indeed the blood samples inoculated with this strain usually became sterile within the 6 hr. period of incubation.

Blood samples from 160 conventionally reared piglets of the same age as those tested against the noncapsulated strain were tested against the capsulated cocci. Sixty-eight (42%) resembled piglet No. 2 (Table 1) in that their blood allowed multiplication. Somewhat unexpectedly, blood from the remaining 92 animals (58%) was bacteriostatic. This difference between animals of the same age group and, indeed, sometimes from the same litter is difficult to explain and will be discussed later. It is possible that the animals whose blood was bacteriostatic were immune as a result either of colostral antibody or of previous infection with *Streptococcus suis*, for when *colostrum-deprived* piglets have been reared under 'pathogen-free' conditions, with 1 exception (see Table 5) their blood was found to lack bacteriostatic power over the capsulated cocci.

The microscopic examination of 'buffy-coat' preparations from the blood of piglet No. 2 inoculated with capsulated and noncapsulated *S. suis* revealed that after 4 hr. rotation at 37°C. the capsulated cocci were proliferating extra-cellularly whereas the noncapsulated organisms had been phagocytosed by polymorphonuclear leucocytes.

It was concluded from these experiments that the possession of a capsule by strain PM1 was one factor concerned in the capacity of this strain for multiplication in piglet blood. It seems likely that in protecting the cocci from phagocytosis the capsule functions in a manner analogous to the capsules of pneumococci and the M antigen of group A streptococci.

Table 2. *Survival of capsulated* Streptococcus suis *in sow and piglet blood*

Source of blood	C.F.U. in inoculum (0·1 ml.) added to blood (0·4 ml.)	C.F.U. in 0·1 ml. of inoculated blood after rotation at 37°C. for stated period				
		0 hr.	2 hr.	4 hr.	6 hr.	24 hr.
Sow	50	12	2	9	18	28
Sow	20	2	2	1	7	0
Piglet (8 days old)	50	6	8	++	++	++
Piglet (8 days old)	20	4	3	144	++	++

Effect of age of the pig on the bacteriostatic power of its blood

In the next experiment the growth of capsulated *S. suis* was compared in blood taken from a piglet and from its dam. A sample of each was inoculated and rotated with *S. suis* as in the previous experiment. Duplicate pour-plates for colony counts were made from 0·1 ml. samples taken before and at intervals during rotation.

Table 2 records the number of colonies developing in the pour-plates from a typical experiment. It can be seen that the capsulated cocci failed to multiply in adult blood but, as in the previous experiment, they grew freely in the piglet blood. Blood samples from four different sows, reared conventionally, were tested in this manner, but in no case did they permit growth of capsulated *S. suis*.

'Buffy-coat' preparations made from adult sow blood and rotated with capsulated organisms showed mainly intracellular cocci. It was concluded that phagocytosis was probably responsible for preventing the growth of these streptococci in adult blood.

The next experiment sought to determine at what age the bacteriostatic property against capsulated *S. suis* appeared in the blood of pigs reared under conventional conditions.

Serial blood samples were taken from two piglets at intervals of from 4 to 28 days up to the age of 53 days (piglet No. 2) and 68 days (piglet No. 228). The samples were tested immediately after venepuncture for their capacity to inhibit the growth of capsulated *S. suis*. The bactericidal tests were performed as already described and although the samples could not be tested simultaneously an attempt was made to keep standard the conditions of the individual tests.

Table 3. *Effect of piglet age on bacteriostatic activity of its blood against capsulated* Streptococcus suis

Serial number of piglet	Age of piglet when tested (days)	C.F.U. in inoculum (0·1 ml.) added to piglet blood (0·4 ml.)	C.F.U. in inoculated blood after rotation at 37°C for stated period	
			0 hr.	6 hr.
2	16	256	1	+ +
	22	330	4	+ +
	32	272	2	+ +
	38	290	3	0
	44	142	1	2
	53	338	6	0
				3 hrs.
228	6	24	6	112
	10	45	11	+ +
	11	50	12	+ +
	20	124	31	+ +
	47	71	18	52
	55	55	14	60
	68	67	17	0

Piglet 2: C.F.U. were estimated from loopfuls of inoculated blood (surface streak).
Piglet 228: C.F.U. were estimated from 0·1 ml. samples of inoculated blood (pour-plates).

The results of several different tests on each piglet are set out in Table 3. It can be seen that the blood of both animals became bacteriostatic between 5 and 7 weeks after birth. Thirteen other piglets, conventionally reared, were tested in the same way and in all the blood became bacteriostatic in from 5 to 8 weeks.

From these experiments it was concluded that during the first weeks of life the blood of about 40% of conventionally reared and a higher proportion of gnotobiotic piglets permits the growth of capsulated *S. suis* when tested *in vitro*. At this age the polymorphonuclear leucocytes appear unable to phagocytose capsulated cocci although they are able to engulf noncapsulated cocci. During the first 5 to 8 weeks of life in a normal environment the piglets become resistant to infection with *S. suis* In the same period their blood becomes bacteriostatic for capsulated

S. suis and their polymorphonuclear leucocytes capable of disposing of these organisms by phagocytosis. It should be mentioned here that, in the case of two piglets delivered by hysterectomy and reared in 'pathogen-free' conditions, the blood had not become bacteriostatic when the final tests were made 156 days after birth. It seemed possible that in conventionally reared animals the bacteriostatic power of the blood resulted from the presence of antibody induced by previous infection with and specifically directed against *S. suis*. Experiments were therefore designed to show whether bacteriostatic activity could be transferred by serum from sow to piglet blood and, if so, whether such activity could be specifically 'blocked' by extracts of *S. suis*.

Capacity of serum from normal and gnotobiotic pigs to produce bacteriostatic activity in piglet blood

An experiment was designed to show whether bacteriostatic activity against capsulated *S. suis* could be transferred from sow to piglet blood by the addition of normal sow serum (Indirect Bactericidal Test).

Table 4. *Bacteriostatic effect of sow serum added to piglet blood inoculated with* Streptococcus suis *and rotated at* 37°*C*

Addition (0·1 ml.) to inoculated piglet blood (0·4 ml.)	C.F.U. in 0·1 ml.* piglet blood after rotation at 37°C. for stated periods	
	3 hr.	4 hr.
Sow serum diluted 1:1	1	0
Sow serum diluted 1:2	0	0
Sow serum diluted 1:5	6	1
Sow serum diluted 1:10	20	76
Sow serum diluted 1:20	380	+ +
Saline	400	+ +
Nil	260	+ +

* The inoculated piglet blood contained 14 C.F.U. in 0·1 ml. before rotation.

A heparinized sample of piglet blood was inoculated with *S. suis* and dispensed in vials in 0·4 ml. amounts. To these were then added 0·1 ml. amounts of normal sow serum serially diluted in normal saline. Control vials received piglet blood either alone or with saline instead of sow serum. The vials were then rotated at 37°C. and sampled at intervals for colony counts in pour-plates.

The results of this experiment are shown in Table 4. It can be seen that the cocci failed to grow freely in blood to which sow serum had been added in dilutions up to 1/10; higher dilutions permitted growth. Serum from five normal sows tested in this way was found to confer bacteriostatic activity when added to piglet blood in dilutions up to 1/10. The same result was achieved with serum from three piglets, 6 to 8 weeks old, whose blood had become bacteriostatic for *S. suis* at that age. On the other hand, serum from seven out of eight gnotobiotic pigs, aged from 11 to 22 weeks, failed to promote bacteriostatic activity when added to normal piglet blood. These results are set out in Table 5.

Table 5. *Effect of serum from conventionally reared and gnotobiotic pigs on growth of* Streptococcus suis *in piglet blood*

Pigs providing serum for test			
Method of Rearing	Age	Number tested	Number of pigs whose serum caused bacteriostasis when added to piglet blood
Conventional	2 years	5	5
Conventional	6–10 weeks	3	3*
Gnotobiotic	22 weeks	2	0
Gnotobiotic	11 weeks	6	1†

* Blood from these pigs did not show bacteriostatic activity against *S. suis* when tested before 5 weeks of age. † Three samples of serum taken from this animal at 1 to 8 weeks of age produced bacteriostasis against capsulated *S. suis* in normal piglet blood.

Table 6. *Effect of* Streptococcus suis *polysaccharides on bacteriostatic effect of normal sow serum added to piglet blood*

Addition made to piglet blood inoculated with *S. suis*	C.F.U. in inoculated blood* after rotation at 37°C. for stated period	
	3 hr.	4 hr.
Sow serum + capsular polysaccharide	138	+ +
Sow serum + cell wall polysaccharide	3	0
Sow serum + broth	15	1
Piglet serum	+ +	+ +

* The inoculated piglet blood contained seven C.F.U. in 0·1 ml. before rotation. The polysaccharides were tested in a final concentration of 0·08 mg/ml. of sow serum.

Specific inhibition of bacteriostatic activity by capsular polysaccharide from Streptococcus suis

It was obviously desirable to know whether the bacteriostatic effect of normal sow serum was specifically directed against *S. suis*. A serum sample was therefore treated with capsular polysaccharide from this micro-organism to see whether the bacteriostatic power was thereby reduced.

A partially purified preparation of capsular polysaccharide from *S. suis* (Elliott, 1966) was dissolved in saline to give a concentration of 0·1 mg./ml. To 0·4 ml. of this solution was added 0·1 ml. of normal sow serum. In control tubes the sow serum was diluted either in nutrient broth or in a saline solution of cell-wall polysaccharide (0·1 mg./ml.) extracted from the noncapsulated strain of *S. suis*, strain A227. Indirect bactericidal tests were set up as in the previous experiment; piglet blood containing capsulated *S. suis* (0·4 ml.) was mixed with 0·1 ml. amounts of serum plus polysaccharide or serum plus broth, rotated at 37°C. and colony counts made in the usual manner.

The results of this experiment are shown in Table 6. It can be seen that capsular polysaccharide from *S. suis* in a final concentration of 0·08 mg. per ml. 'blocked' the bacteriostatic activity of sow serum added to piglet blood. Cell-wall polysaccharide from the noncapsulated variant failed to do so.

The results of this experiment strongly suggested that inhibition of the growth of *S. suis* in the blood of conventionally reared pigs was promoted by antibody specifically directed towards the capsules of these micro-organisms. Efforts to demonstrate such antibody by *in vitro* methods were unsuccessful; the methods employed included precipitation, agglutination, complement fixation and anti-globulin tests. An experiment was therefore designed to show whether antibody could be demonstrated in normal sow serum by its specific protection of susceptible piglets against experimental infection with *S. suis*.

Protective effect of normal sow serum against Streptococcus suis *infection in piglets*

Elliott *et al.* (1966) showed that bacteriaemia can be produced in susceptible piglets by spraying broth cultures of *S. suis* into the nasopharynx and that such infection can be prevented by the prior administration of serum from piglets convalescent from experimental infection. In the protection experiment now to be described 28 piglets were divided equally into two groups. The animals in one group had received subcutaneously 20 ml. of normal sow serum. Twenty-four hours later 5 ml. of an overnight broth culture of *S. suis* was sprayed into the nasopharynx of each of the 28 animals comprising both groups. Blood cultures were made from all the animals 2 days later and thereafter periodically up to 7 days.

Of the 14 animals that had received normal sow serum only one developed a bacteriaemia within 7 days of spraying. Of the 14 that received no serum, ten developed a *S. suis* bacteriaemia within 7 days of experimental infection.

Separation of bacteriostatic component from normal sow serum

The experimental evidence here presented strongly suggested that the resistance of adult pigs to *S. suis* infection resulted from active immunity following subclinical infection contracted in early life. The circulating antibody responsible appeared to be directed specifically against the capsular polysaccharide of *S. suis*. It was present in the blood in concentrations sufficient for detection by protection tests or by the bactericidal test but too small for recognition by the other *in vitro* serological tests employed. We therefore attempted to separate the responsible component in pig serum by fractionation procedures such as would enable us to compare its physical properties with those of the known immunoglobulins.

With this object in view 550 ml. of normal sow serum was treated in the manner shown in Fig. 1. Details of the procedures used are given below and under Materials and Methods.

Step 1. *Euglobulin precipitation.* Serum from a 2-year-old sow was separated into euglobin and pseudoglobulin fractions by dialysis against distilled water. Both fractions were made to the original volume of serum and tested at a dilution of 1/25 for bacteriostatic activity (Table 7). Only the pseudoglobulin fraction showed the activity at this dilution. The euglobulin showed activity undiluted and at a 1/10 dilution.

Step 2. *Ammonium sulphate precipitation.* The pseudoglobulin fraction was brought to 0·3 saturation with solid ammonium sulphate in the cold. The precipi-

tate (0·3 fraction) was centrifuged, re-dissolved in water and dialysed against physiological saline. The supernatant was brought to 0·5 saturation by the addition of further ammonium sulphate and a 0·3–0·5 fraction collected. The second supernatant was brought to 0·7 saturation and a 0·5–0·7 fraction obtained. The final supernatant was discarded.

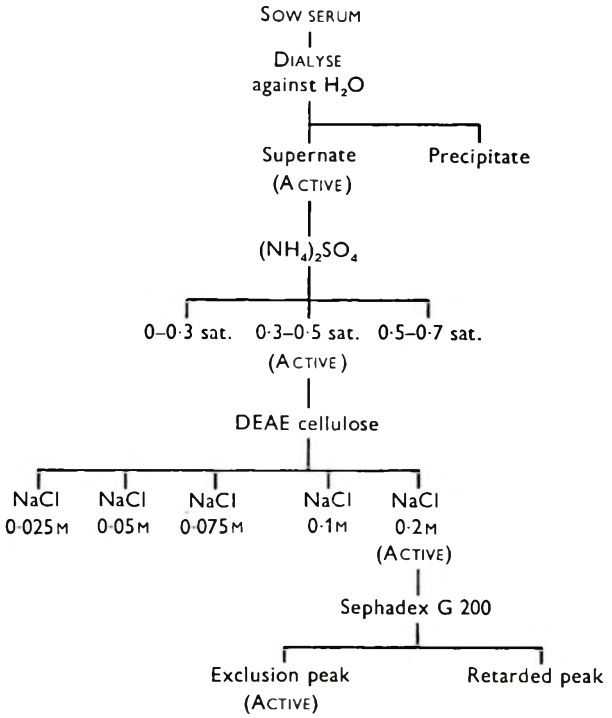

Fig. 1. Separation of bacteriostatic component from sow serum.

The three fractions were tested for bacteriostatic activity at a dilution corresponding to 1/10 of the initial pseudoglobulin concentration (Table 7). Only the 0·3–0·5 saturation fraction showed activity. Roughly 6·5 g. of this fraction were recovered from 550 ml. serum (approximately 12 mg./ml.)

Step 3. *Ion exchange chromatography on DEAE cellulose.* The 0·3–0·5 saturation ammonium sulphate fraction from pig pseudoglobulin was further fractionated on DEAE cellulose as described under Materials and Methods. Roughly 4 g. protein was applied to a column containing approximately 800 ml. ion exchanger. After the exclusion peak had been collected, step-wise elution was performed with starting buffer containing NaCl to a final concentration of 0·025 M, 0·05 M, 0·075 M, 0·1 M and 0·2 M, successively.

The eluted fractions were pooled, precipitated by half saturation with ammonium sulphate, dissolved in water and dialysed against physiological saline. The final volume of each fraction was approximately 20 ml., corresponding roughly to a 25-fold concentration of the serum. The fractions were tested for bactericidal activity diluted 1/20. As may be seen in Table 7, only the 0·2 M NaCl fraction showed inhibitory activity in these conditions. The protein concentration of this fraction

was approximately 30 mg./ml. Roughly 600 mg. of this fraction was recovered from 550 ml. serum (approximately 1 mg./ml.).

Step 4. *Gel filtration on G*200 *Sephadex.* Ten ml. of the 0·2 M-NaCl fraction from DEAE fractionation were applied to a 2 l. G 200 Sephadex column. Blue dextran and haemoglobulin were added as markers. The elution pattern is shown in Fig. 2. Fractions were pooled as shown and concentrated by ammonium sulphate precipitation as before. It will be seen from Table 7 that only the exclusion peak showed bacteriostatic activity.

Table 7. *Bacteriostatic effect of sow serum fractions added to piglet blood inoculated with* Streptococcus suis

Fractionation of sow serum	Serum fraction (0·1 ml.) added to inoculated piglet blood (0·4 ml.)	C.F.U. in 0·1 ml. piglet blood after rotation at 37°C. for stated period		
		0 hr.	3 hr.	4 hr.
Step 1				
Dialysis V.H_2O	Supernate dil. 1 = 25 (pseudoglobulin)	11	10	6
	Precipitate dil. 1 = 25 (euglobulin)		117	++
Step 2				
$(NH_4)_2SO_4$ added to supernate from step 1	Precip. from 0·3 sat. (Fract. 1)	10	9	155
	Precip. from 0·3 to 0·5 sat. (Fract. 2)		0	0
	Precip. from 0·5 to 0·7 sat. (Fract. 3)		35	230
Step 3				
DEAE cellulose treatment of fract. 2 from step 2	Peak 1 eluted by buffer	16	40	136
	Peak 2 eluted by NaCl 0·025 M		12	155
	Peak 3 eluted by NaCl 0·05 M		12	++
	Peak 4 eluted by NaCl 0·075 M		46	75
	Peak 5 eluted by NaCl 0·1 M		10	173
	Peak 6 eluted by NaCl 0·2 M		1	3
Step 4				
Gel filtration* of peak 6 from step 3	Exclusion peak	8	4	5
	Retarded peak		23	275

Appropriate controls were included in all tests but have been omitted from Table 7 because inactive fractions also served as 'negative' controls. * Gel filtration was carried out using Sephadex G 200.

Effect of 2*-mercaptoethanol on bacteriostatic activity of sow serum*

Unfractionated sow serum and the bacteriostatic component obtained by Sephadex filtration of the active fraction above described were mixed each with an equal volume of 0·2 M 2-mercaptoethanol and incubated at 37°C. for 2 hr. The mercaptoethanol was then removed by prolonged dialysis against normal saline. The nondialysable residue was sterilized by filtration and tested for bacteriostatic activity against capsulated *S. suis* in piglet blood.

The results of this experiment are shown in Table 8, from which it can be seen that the bacteriostatic activity of both the unfractionated sow serum and the

active component obtained by gel-filtration were destroyed by the mercaptoethanol treatment, thus confirming the provisional identification of an IgM immunoglobulin as the active component.

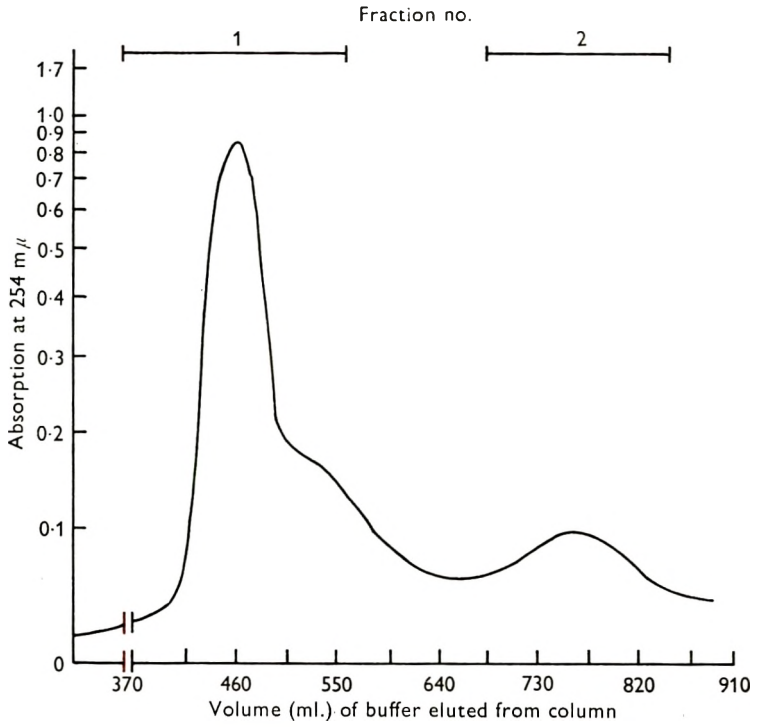

Fig. 2. G 200 Sephadex gel filtration of active fraction from DEAE cellulose column.

Table 8. *Effect of 2-mercaptoethanol on bacteriostatic activity of sow serum and its active component separated by gel filtration**

Addition (0·1 ml.) to piglet blood inoculated with *S. suis* (0·4 ml.)	C.F.U. in 0·1 ml. of inoculated blood after rotation at 37°C. for stated period	
	3 hr.	4 hr.
Sow serum before SH treatment	7	4
Sow serum after SH treatment	74	+ +
* Gel filtration exclus. peak before SH treatment	1	5
* Gel filtration exclus. peak after SH treatment	42	151
Saline	130	+ +

* See step 4, Table 7. SH = 2 Mercaptoethanol (0·2 M). The inoculated piglet blood contained 14 C.F.U. in 0·1 ml. before inoculation.

DISCUSSION

Streptococci cause neonatal infections in a variety of domesticated animals including man, (Eickhoff *et al.* 1964), horses (Gunning, 1947), sheep and pigs. The micro-organisms responsible for these infections of the newborn rarely cause

disease in adults of the same species and they are usually without virulence for laboratory animals. Evidence presented here suggests that in pigs specific immunity is a factor in adult insusceptibility and that such immunity may result from inapparent infections of early life.

S. suis is the usual cause of streptococcal infection in piglets. Adult pigs are insusceptible to experimental infection with these micro-organisms, whereas piglets within the first 4 weeks of life can be infected by spraying broth cultures into the nasopharynx. We have shown that with increasing age the blood of piglets becomes inhibitory to the growth of *S. suis* and it seems reasonable to suppose that this is associated with decreasing susceptibility to infection. The bacteriostatic action of the blood results from phagocytic activity mediated by a serum component directed specifically against the capsular polysaccharide of the streptococci.

Such evidence as we have been able to gather from the behaviour of this component under the fractionation procedure applied to pig serum suggests that it belongs to the IgM class of macroglobulins. Its presence in greater part in the pseudoglobulin fraction of the pig serum was an unexpected finding but its behaviour on Sephadex and DEAE cellulose and the destruction of its bactericidal activity by mercaptoethanol confirms its identification as IgM. We cannot say with certainty that this antibody results from previous infection with *S. suis*, but three facts support such a possiblity: first, there is evidence that of normal sows at least 7 % carry *S. suis* in the upper respiratory tract (Elliott *et al.* 1966); secondly, the same investigators found that in naturally occurring outbreaks of *S. suis* infection, throat cultures reveal all piglets of a litter to be infected although a minority may show signs of overt disease; and finally, in the present investigation three out of four pigs reared under pathogen-free conditions failed by the age of 6 months to develop bacteriostatic activity in their blood against *S. suis*; in our experience pigs reared under conventional conditions develop such activity within the first 8 weeks of life. From these considerations it seems likely that, where *S. suis* is concerned, inapparent infections may greatly exceed in number those causing overt diseases and may be the source of the specific immunity of adult pigs.

We have no explanation to offer for our observation that, of the normal piglets examined within 3 weeks of birth, the blood of nearly 60 % was bacteriostatic for *S. suis*. Presumably these were immune to infection. Sometimes piglets with and without bacteriostatic activity were found in the same litter. We have not examined colostrum for bacteriostatic antibody although this might be one source of passive immunity during the first weeks of life. It seems likely that with respect to the amount of colostrum received all piglets in a litter are not equal.

SUMMARY

Phagocytosis prevents the multiplication of *Streptococcus suis* in blood samples from conventionally reared adult pigs. It is mediated by antibody, probably IgM, present in the serum of adults and specifically directed against the capsular polysaccharide of *S. suis*. The blood of young pigs reared in a 'pathogen-free' environ-

ment allows multiplication of encapsulated *S. suis*. Of 160 piglets conventionally reared, the blood of 92 (58 %) did not permit multiplication of *S. suis*. Blood from the remaining 68 (42 %) allowed multiplication but became bacteriostatic when the animals reached the age of 6 to 8 weeks. Serum from adult pigs confers bacteriostatic activity on blood from susceptible piglets *in vitro* and passively protects such piglets against experimental *S. suis* infection *in vivo*.

We thank Dr T. J. L. Alexander, formerly of Ontario Veterinary College, and Mr P. C. Trexler of the Royal Veterinary College, London, for providing us with serum from gnotobiotic pigs.

The work described in this report was carried out by one of us (K. K. A.) during the tenure of a Commonwealth Scholarship and was incorporated in a dissertation submitted for the Ph.D. degree of the University of Cambridge.

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Salmonella excretion by pet terrapins

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INTRODUCTION

Salmonellas were first isolated from turtles by McNeil & Hinshaw (1946) and from tortoises by Boycott, Taylor & Douglas (1953) who recognized the risk of spread of infection from these animals to man. Probably the first recorded finding of a salmonella in a small aquatic turtle (which would be called a terrapin in Britain) was in Florida between 1949 and 1953 (Report, 1962).

Seventeen years after the original observation the first case of human salmonellosis with a terrapin as source was reported (Hersey & Mason, 1963) and since then over fifty American cases which were epidemiologically associated with terrapins have been described (Kaufmann & Morrison, 1966). In the United Kingdom Plows, Fretwell & Parry (1968) have very recently recorded the infection of a patient with an *Arizona* species which came from an infected terrapin. They also isolated several salmonellas from one of the patient's terrapins, and refer to a case of salmonellosis in Liverpool where the most likely source was again a terrapin.

We wish to bring to notice that pet terrapins in this country may be infected with salmonellas and suggest that this be borne in mind when any case of salmonellosis is investigated.

A four-year-old boy living in the area served by this laboratory developed gastro-enteritis due to *Salmonella paratyphi B* phage type Battersea. Subsequent enquiries revealed that there were three pet terrapins kept in his home and that he had been in the habit of playing with them. The water in which they lived was cultured and found to contain *Salmonella paratyphi B* phage type Battersea. Each of the three terrapins was subsequently shown to be excreting *S. paratyphi B* phage type Battersea. As a result of these findings we examined terrapins and water from terrapins' tanks taken from pet stores, schools and private houses in the same district.

MATERIALS AND METHODS

Samples of tank water were inoculated directly on brilliant green MacConkey agar (Harvey, 1956) and deoxycholate citrate agar (DCA) plates and were also mixed with equal volumes of selenite F medium and incubated at 37° C. Subcultures were made after 1 and 5 days on DCA and brilliant green MacConkey agar. All plates were incubated overnight. Any non-lactose-fermenting colonies were subcultured and identified by the usual serological and biochemical tests. Each terrapin received was washed and placed in a sterile bowl filled with tap water and kept for 1 week. Samples of the water were taken at intervals and were examined as described. After 5–7 days each terrapin was killed with nembutal. Its viscera were removed and macerated, and were added to selenite F and nutrient broths and incubated at 37° C. After 1 and 5 days subcultures were made and examined.

RESULTS

Water samples taken from 21 tanks in schools, shops or homes, each containing one or more terrapins, were examined. Only four of these yielded salmonellas and one was water from the tank containing the terrapins of the index case. Salmonellas isolated were: *S. paratyphi B* phage type Battersea (from two tanks), *S. madelia* and *S. houten*.

Table 1. *Details of isolations from infected terrapins*

Terrapin Identification no.	Organism excreted during life in laboratory	Organism isolated only from viscera
1	*S. paratyphi B*, phage type Battersea	—
2	*S. paratyphi B*, phage type Battersea	—
3	*S. paratyphi B*, phage type Battersea	—
4	—	*S. paratyphi B*, phage type Battersea
6	*S. paratyphi B*, phage type Battersea	—
9	*Arizona* sp	—
10	*S. urbana*	—
19*	—	*Arizona* sp
27	*S. newport*, *Arizona* sp	—
28*	—	*S. newport*, *S. panama*, *Arizona* sp
29	*S. madelia*	—
30	*S. newport*	—
31	*S. newport*	—
32	—	*S. saintpaul*, *S. infantis*, *Arizona* sp
33	—	*S. madelia*, *S. saintpaul*
34	*S. heidelberg*	—
38	—	*Arizona* sp

* These animals were received dead. It was not possible to study their excretion in life.

Thirty-nine terrapins were sent to the laboratory for examination. Of these 14 were found to be carrying salmonellas and six to be carrying members of the closely related *Arizona* species. Most carried only one organism but two of the creatures each carried two different salmonellas and an arizona, one carried two salmonellas and one a salmonella and an arizona. The majority of the terrapins

excreted their organisms into the bowl of water in which they were kept in the laboratory but a minority did not; the organisms that they carried were only demonstrated when their viscera were cultured. The exact distribution of the organisms found is given in Table 1.

DISCUSSION

Terrapins are well known to carry salmonellas and thus to be a source of human salmonellosis (Kaufmann & Morrison, 1966). Indeed Williams & Helsdon (1965) considered them such an important source of certain salmonellas as to refer to these types as 'terrapin associated'. Reardon & Wilder (1964) were sufficiently impressed with this association to look specifically, and successfully, for a terrapin source whenever a certain serotype (in their case they chose *S. braenderup*) was found in children. For similar reasons Rosenstein, Russo & Hinchliffe (1965) sought and found a terrapin source in an outbreak they were investigating.

Terrapins are imported into the United Kingdom mainly from the United States. They are bred in captivity in large 'farms' in Louisiana and Mississippi, and are fed on rendered meat scraps (Ager, 1963). Investigations at one such 'farm' (Quist & McQueen, 1963) revealed that 2 out of 10 adult terrapins carried salmonellas and at another farm Kaufmann & Morrison (1966) demonstrated salmonellas in the feed stuff, pool water and soil around the bank and also in the ovaries, gallbladder and eggs of the terrapins themselves. The water in these farms was not potable. Moreover meat scraps have been shown to contain salmonellas (Ager, 1963). It was therefore suggested that water and meat scraps were the sources of contamination. It should be remembered, however, that reptiles as a group (both wild and captive) frequently harbour salmonellas (Hinshaw & McNeil, 1945, 1947; Lee & Mackerras, 1955). Thus it may be that the terrapins themselves are the source of the salmonellas. Certainly Kaufmann & Morrison (1966) have demonstrated means by which the female could infect her own young and so perpetuate infection in the species.

Now that the problem has become known and in response to suggestions for salmonella-free terrapins (Kaufmann & Morrison, 1966; Williams & Helsdon, 1965) the more responsible breeders are taking steps to avoid contamination of their stocks by using salmonella-free foods and chlorinated water where possible, and by generally increasing their hygienic standards. However, many of the salmonella types we have isolated are uncommon in this country and this suggests that they are still being imported with the terrapins.

After their arrival in the United Kingdom the terrapins are fed by the larger dealers on raw meat scraps, earthworms and tubifex worms. Meat scraps are a potent source of salmonellas and tubifex worms from river mud may also be contaminated with salmonellas, as river sludge is a known source of salmonellas (Taylor *et al.* 1965). These represent other ways in which infection may be introduced or maintained in a terrapin population. The dried insect foods used by the smaller dealers and in the home seem to be safer, however. We have examined ten samples of these and have isolated no pathogens. Other workers (Williams & Helsdon, 1965; Rosenstein *et al.* 1965; Reardon & Wilder, 1964) report similar

negative findings. Kaufmann, Feeley & De Witt (1967) have shown that small batches of terrapins will remain infected for at least a year and we found two terrapins to be excreting after a year's captivity in a Sheffield household. Thus despite Williams & Helsdon's (1965) observation that the proportion of terrapins infected falls with the time of existence away from their hatchery it should not be assumed that they will all become infection-free after a given period.

We have examined the terrapins by culture of their environmental water and of their viscera and we report salmonellas isolated from both materials. It could be argued that only salmonellas present in the water represent a hazard. However, Kaufmann *et al.* (1967) have shown that some terrapins contaminated their water only intermittently and so a negative water sample is no guarantee that the water may not become infected later. For this reason we consider that all carrier terrapins may be dangerous and accordingly we report any salmonellas isolated from the animal as well as its water.

In our survey we detected salmonellas in only four of twenty-one specimens of water from tanks in the owners' homes. This was much less than the proportion of positive results from the water in the bowls of the terrapins which we kept in the laboratory. This may well have been due to the larger volumes of water used in home aquaria and also to the lack of water changes while the terrapins were kept in the laboratory, whereas in the home water is changed every 2–3 days. Negative results from occasional tank-water testing are therefore no guarantee that a terrapin is not a salmonella carrier. Our findings indicate that a considerable proportion of the terrapins in this country may be expected to be carrying salmonellas, and the case we report demonstrates forcibly that these creatures represent a health hazard to the community.

All the creatures that we examined were red-eared turtles (*Pseudemys scripta elegans*) but this is not the only species which has been shown to be infected (Plows *et al.* 1968; Williams & Helsdon, 1965). The related land turtle, the tortoise, has been shown on many occasions in this and many other countries to carry salmonellas (Boycott *et al.* 1953; Douglas & Taylor, 1954; Kaufmann & Morrison, 1966). The tortoise has been implicated as the source of human infection (Ludkin, 1955) but it is rarely so because its infected excreta are unlikely to find their way into human mouths. The terrapin, in contrast, excretes salmonellas into a warm fluid environment, in which the organisms will survive well or even multiply. Moreover this infected fluid is situated inside the house where it is very likely to contaminate hands or food, more especially if water changes are carried out (as they usually are) in the kitchen sink.

It is easy to see from this how dangerous this seemingly very clean pet may be and how easily its organisms may be spread in the home. If these animals are to be kept there, we think that considerable care should be taken to reduce the risk of infection to the family. Sensible control measures have been recommended to persons possessing pet terrapins by Williams & Helsdon (1965). These are: (1) children should not be allowed to handle turtles unless they are responsible enough to wash their hands after contact; (2) turtle water should not be discharged into the kitchen sink or allowed to contaminate the food preparation area; (3) a

special container should be designated as the turtle dish and be used for nothing else; (4) only one person who is careful to wash his hands should care for the turtle and (5) other household pets should be prevented from drinking the water from the turtle dish.

It is current practice in some infant schools to keep terrapins for the children's interest and observation. Here, too, is a very distinct risk of children being infected, especially if they are allowed to play with the terrapins. Strict precautions should be taken to prevent contamination of fingers. Similarly, the staff of pet stores must also be at risk and this must underline the importance of applying the provisions of the Offices, Shops and Railway Premises Act which is designed, among other things, to protect personnel from such hazards.

We hope that this account will alert those who keep terrapins to their dangers and suggest that simple hygienic measures, to prevent contamination of hands, kitchen and food, are instituted. We hope, also, that it will serve to keep in the mind of any worker investigating an outbreak of salmonellosis that a terrapin may be the source of infection.

SUMMARY

The infection of a child with *Salmonella paratyphi B* phage type Battersea caught from his pet terrapins is recorded. In a small subsequent survey a relatively large proportion of these animals was found to be excreting salmonellas. The danger of these seemingly harmless pets is emphasized.

It is a pleasure to acknowledge the help and encouragement given by Dr E. H. Gillespie at all stages in the production of this manuscript. We also thank Dr Joan Taylor and Dr J. Wallace for final identification of some of the organisms isolated and Dr E. S. Anderson for phage typing the *Salmonella paratyphi B* strains.

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Pantothenate-requiring dwarf colony variants of *Staphylococcus aureus* as the etiological agent in bovine mastitis

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INTRODUCTION

A number of authors have described dwarf colony variants of *Staphylococcus aureus* (D forms) obtained from normal strains kept under adverse conditions, and in a few cases such variants have been isolated from infectious lesions; in the latter cases the cultures developed normal colonies when incubated under increased CO_2 pressure. The metabolic defect causing the unusual growth feature had not been identified in any case (for references see Sompolinsky, Ernst-Geller & Segal, 1967).

As far as we are aware, D forms of *S. aureus* as the etiological agent in bovine mastitis have hitherto only been described in Israel. Since our earlier communication (Lernau & Sompolinsky, 1962) mastitis due to D staphylococci has been observed in nearly 30 herds under intensive mastitis control programme, which is more than 35 % of the herds under this programme, and also in a few other herds. D staphylococci have not been isolated from ovine mastitis (Dr R. Tamarin, personal communication) nor have they been observed among more than 150,000 strains of human origin examined by the Staphylococcus Reference Laboratory of Israel.

Most of the D strains isolated were thiamineless, i.e. when thiamine HCl was added to sterile nutrient media the bacteria grew as normal colonies. Two distinct metabolic disorders have been observed in the thiamineless strains: (1) inability to concentrate the thiazole moiety of thiamine; and (2) inability to phosphorylate thiamine-pyrimidine (Sompolinsky *et al.* 1967). In two herds D strains with other metabolic disorders have been isolated. In this paper we shall describe a group of strains isolated from one herd, and discuss a few epidemiological observations of the infection.

MATERIALS AND METHODS

Milk samples were plated on Difco blood agar base enriched with 4·0 % washed sheep erythrocytes. *Streptococcus agalactiae* was used to potentiate the staphylococcal β-haemolysin in the warm phase incubation (camp reaction).

The isolated staphylococcal strains were cultivated on Difco nutrient agar, Difco tryptose phosphate agar and sheep blood agar; the last medium was composed

of beef broth essentially as described (Sompolinsky, Saruf & Glazewski, 1966) enriched with 4% washed sheep erythrocytes and solidified with Bacto agar.

The basic medium for examinations of vitamin requirements was prepared on the basis of vitamin-free casamino acids (Difco), purified with active carbon: Bacto vitamin-free casamino acids (purified), 25 g.; NaCl, 8 g.; KH_2PO_4, 1·2 g.; K_2HPO_4, 1·2 g.; Special Noble agar, Difco, 15 g.; distilled water to 1 l.; pH 7·2.

To this medium was added sterile solutions of thiamine HCl, nicotinic acid, biotine and L-cysteine to final concentrations of 10^{-6} (w/v) each. This medium will be designated CVF. Other supplements will be specified under Results. Most growth factors were purchased from Sigma Chemical Co. Pantoic acid was prepared by alkaline hydrolysis of DL-pantoyl lactone as indicated by Grula & Grula (1962).

RESULTS

Epidemiological observations

When dwarf staphylococci were first isolated, the herd consisted of 55 lactating cows, 28 of which were infected with *S. aureus* in at least one quarter of the udder. The udders from which D strains were isolated showed upon palpation a marked degree of parenchymal fibrosis, indicating a rather chronic state of infection. Before the first isolation of the D staphylococci, the infected cows were treated on several occasions by the intra-mammary route with penicillin G, streptomycin, neomycin and tetracycline. The staphylococcal variants to be described were isolated from four cows in January and February 1967, and at each subsequent examination of milk samples from the infected udder quarters until the cows were sold, in one cow (Geveret) during more than one year. When the mastitis milks were plated a pure culture of D colonies was obtained from some samples; others showed a few normal colonies among the dwarfs (Plate 1, Fig. 1).

In our experience, treatment with an antibiotic shown to be suitable by *in vitro* tests is successful in curing glands chronically infected with normal *S. aureus* in about 45% of cases. However, none of the glands infected with D-staphylococci were cured by similar treatment; this is in accordance with our experience with dwarf-staphylococcal mastitis in other herds.

Bacteriological examinations

Eight strains of *S. aureus*, isolated from four cows between January 1967 and February 1968 were included in this study (Table 1). These strains, with the exception of No. 164, grew as pin-point, transparent streptococcus-like colonies on nutrient agar, tryptose phosphate agar and sheep blood agar as well as many other nutrient media in general use. No. 164 produced colonies of almost normal size on these media (Plate 1, Fig. 2) but in spite of this, it was included in this study, since no growth was obtained on unsupplemented CVF and normal colonies developed on CVF + Ca-pantothenate (Table 1). No. 287 was also distinct from the other strains in that it grew with D colonies both on unsupplemented and on pantothenate-CVF. The colonies of No. 207 on sheep blood agar were smaller than those of the other strains and barely visible after 48 hr.

The microscopic morphology was characteristic for staphylococci in all the strains; they produced a wide zone of hot-cold haemolysis on sheep blood agar; the coagulase test was positive; they were negative for Tween-splitting; they were all susceptible only to phage 42E of the international set of typing phages; and all were susceptible to sulphathiazole, penicillin G, streptomycin, tetracycline,

Table 1. *Characterization of eight staphylococcal strains from bovine mastitis in one herd*

	Strains							
				Geveret				
Nutrient medium	Kafrit 164	Timna 207	Mafliga 266	195 RR*	287 RR	348 LR*	806 RR	1131 RR
Tryptose phosphate agar	N†	D†	D	D	D	D	D	D
Sheep blood agar	N	D	D	D	D	D	D	D
Vitamin-free Casamino agar (CVF)	Neg†	Neg	Neg	Neg	D	Neg	Neg	Neg
CVF + Ca-pantothenate	N	n†	N	N	D	N	N	N

* RR = right rear gland; LR = left rear gland of the udder.

† N = normal colonies. D = dwarf colonies. Neg = no growth.
n = colonies noticeably smaller than those of other pantothenateless strains on medium with equal concentrations of the vitamin.

Table 2. *Growth of D staphylococci on Ca-pantothenate and related compounds*

		Strains							
					Geveret				
Growth factor	Concentration*	Kafrit 164	Timna 207	Mafliga 266	195	287	348	806	1131
D-Ca-pantothenate	10^{-7}	+ +	+	+ +	+ +	D+	+ +	+ +	+ +
	10^{-9}	+	−	+	+	D	N.E.	+	+
β-alanine	10^{-4}	−	−	−	−	D	−	−	−
Pantoic acid	10^{-5}	+	+	+	+	D	+	+	+
DL-pantoyl lactone	10^{-4}	+	D	+	+	D	+	+	+
α-keto-iso-valeric acid	10^{-4}	−	−	−	−	D	−	−	−
D-pantothenyl alcohol	10^{-4}	D	−	+	−	D	−	−	−
D-pantethine	10^{-4}	+ +	+	+ +	+ +	D	+ +	+ +	+ +
	10^{-6}	+	−	+	+	D	+	+	+

* in w/v of the vitamin-free casamino agar medium.

+ +, growth of normal colonies in 24 hr.; +, growth of normal or sub-normal colonies in 72 hr.; D, growth of dwarf colonies; −, no growth; N.E., not examined.

chloramphenicol and erythromycin according to a diffusion test with sensitivity tablets (Rosco, Denmark) (Sompolinsky & Minkowski, 1969). After 48–72 hr. incubation on sheep blood agar, normal-sized colonies developed as papillae on some of the D-colonies. From these papillae, prototrophic, coagulase positive strains were isolated, which were susceptible to the same chemotherapeutic agents and typing phages as the progenitor strains.

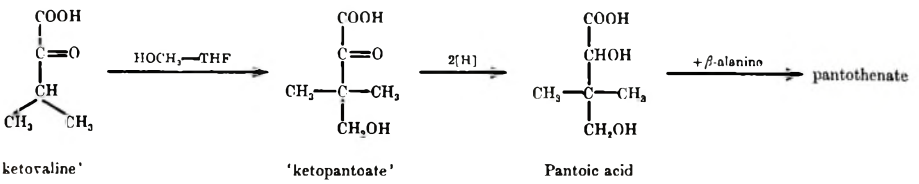
None of the strains responded to the following supplements to CVF: Thiamine

monophosphate (highest concentration tested 10^{-4}, w/v); thiamine pyrophosphate (10^{-4}); vitamin B_{12} (10^{-8}); nicotinamide (10^{-6}); vitamin K_1 (10^{-8}); ascorbic acid (10^{-7}); pyridoxal-HCl (10^{-5}); pyridoxamine di-HCl (10^{-7}); *p*-amino benzoic acid (10^{-7}); thioctic acid (10^{-4}); *i*-inositol (10^{-6}); and DL-threonine (10^{-4}). On CVF supplemented with Ca-pantothenate all strains, except No. 287, developed normal sized staphylococcal colonies in 24 hr (37°). In Table 2, the limiting concentration of pantothenate and response to related compounds is reported. As shown in the Table, the pantothenate-less strains responded also to pantethine (bis-(N-pantothenyl-amidoethyl) disulfide), DL-pantoyl lactone and pantoic acid (α-γ-hydroxy-β,β-dimethyl-butyric acid), prepared by alkaline hydrolysis of the pantoyl lactone. The concentration of pantoyl lactone required for delayed growth was a thousand times, and of pantoic acid a hundred times that of the concentration of Ca-pantothenate giving prompt growth (Plate 2). This may be due to differences in the speed of penetration through the cell membrane, which may also be responsible for the high concentration of pantethine required.

DISCUSSION

Wild strains of *Staphylococcus aureus* generally require the following vitamins for growth: thiamine or its thiazole + pyrimidine moieties, nicotinamide or nicotinic acid (Knight, 1937), and, for some strains, biotine. Requirement for Ca-pantothenate has hitherto not been observed in strains obtained from disease processes.

The biosynthesis of pantothenate in bacteria may be outlined as follows (Davis *et al.* 1968):



The pantothenate is subsequently condensed with cysteine to pantothenyl-cysteine from which pantethine is formed by release of CO_2.

The pantothenateless staphylococcal strains under consideration must be unable to add hydroxymethyl to 'ketovaline' or to reduce 'ketopantoate'. Since this latter compound was not available, we were unable to demonstrate which of these functions was affected.

The frequent occurrence of auxotrophic staphylococci in connexion with bovine mastitis in Israel is not easily explained. The possibility that a single auxotrophic bacterial cell arose by chance, multiplied in an udder gland and was then spread to other cows in the herd by the usual vehicles of infection, is unlikely in the case reported, since the pantothenate-requiring strains from different cows were not similar. No. 164 was distinguished from the other strains by its ability to develop almost normal colonies on tryptose phosphate and on sheep blood agar; any

unknown compound in these media might be used by this strain for production of pantothenate. Strain No. 266 from another cow seemed also to be specific in its ability to grow on pantothenyl alcohol, probably oxidizing it to pantothenate. No. 287, though isolated from the same udder gland as pantothenate-auxotrophs, required another compound that we so far have been unable to specify.

An alternative possibility for the simultaneous occurrence of a number of infections with D staphylococci in mastitis herds might be a selective advantage for the cocci with a specific metabolic disorder. This might be an *in vivo* analogy of 'penicillin-screening'. The fact that antibiotic treatment has always proved unsuccessful in infections with this kind of staphylococci might sustain this hypothesis, but we have so far no real experimental data supporting it further. In every case, some local factor, possibly an unknown mutagenic agent in the milk, must be an additional precondition for the development of these udder infections since they have not been described in other countries, though treatment of mastitis is similar in most areas of intensive milk production.

SUMMARY

In a herd affected with bovine mastitis, dwarf colony variants of *Staphylococcus aureus*, auxotrophic for Ca-pantothenate, were isolated from the milk of four cows with mastitis. From one of these cows, a dwarf variant with an unknown metabolic disorder was isolated during the same period. Infections with auxotrophic staphylococci were always chronic and proved to be refractory to antibiotic treatment, though the causative micro-organism was highly susceptible to the drugs used as judged by *in vitro* tests.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Plating of mastitis milk sample yielding D variant colonies of *Staphylococcus aureus* interspaced with a few normally sized colonies.

Fig. 2. Two pantothenate-less strains of *Staphylococcus aureus* on sheep blood agar (No. 1131 with pin-point colonies and No. 164 with almost normal colonies).

PLATE 2

Subcultures of the dwarf *Staphylococcus aureus*, strain Geveret 195, on: A, plain casamino acid medium (CVF); B, CVF + DL-pantoyl lactone (10^{-5}, w/v); C, CVF + pantoic acid (same conc.); and D, CVF + Ca-pantothenate (10^{-8}). Incubation: 48 hr. at 37° C.

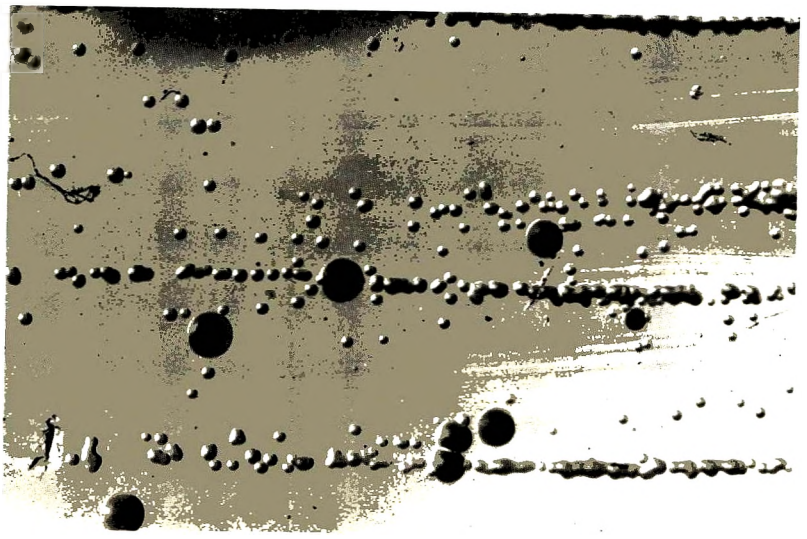


Fig. 1

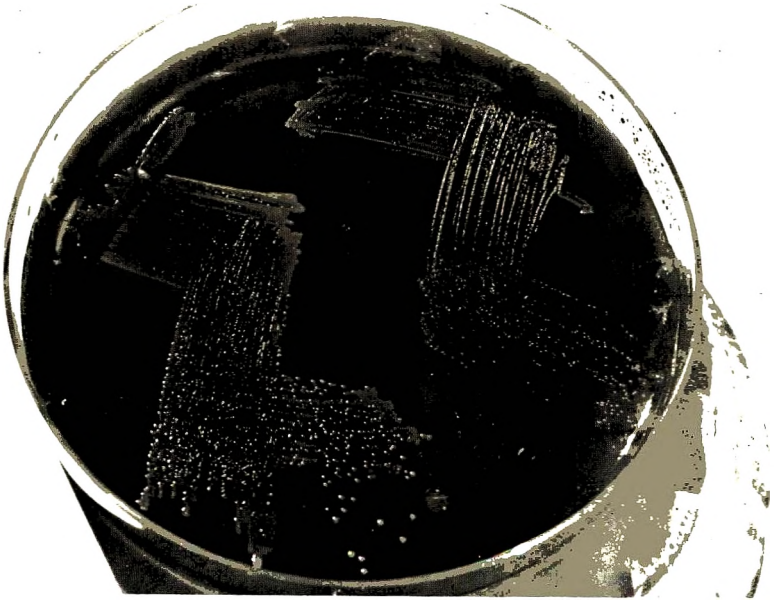
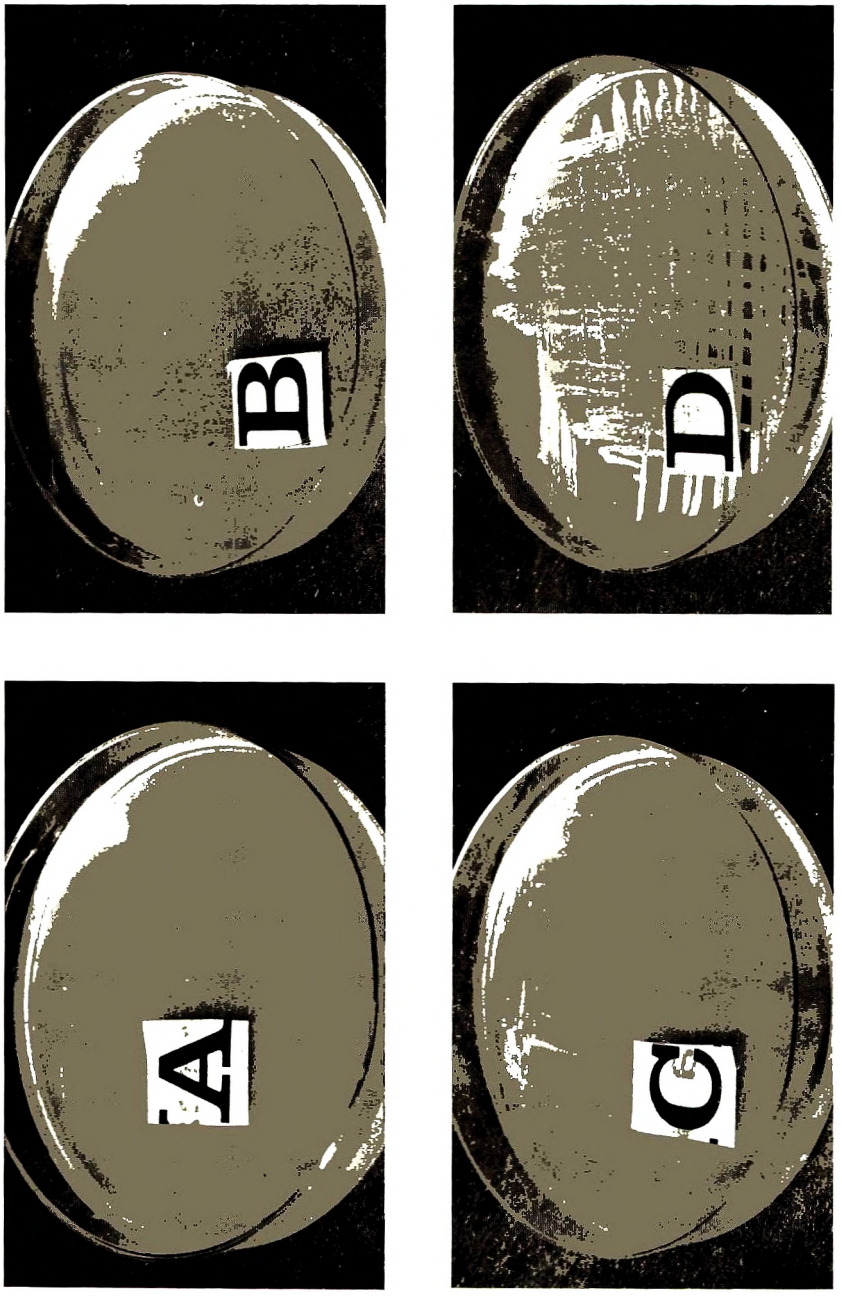


Fig. 2


B
D
A
C

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Salmonellas in sewage. A study in latent human infection

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INTRODUCTION

The salmonella group includes a great many organisms differing in antigenic composition, sometimes in biochemical reactions and often in pathogenicity for man. It is this latter property which probably determines the range of serotypes causing clinical symptoms in human infection. In the present study we have monitored the salmonellas excreted in human sewage irrespective of their ability to cause recognizable illness. In the area selected, general practitioners were asked to look out for and to investigate cases of gastro-enteritis. Those notified during the investigation, however, were not caused by salmonella infection. The current survey was derived from an earlier series of observations on a naturally contaminated river—the river Taff. This study has been briefly described from the technical aspect (Harvey & Price, 1967*a*). The investigation of human sewage was decided on to ascertain the probable source of frequent isolations of salmonellas from river water. The estate chosen for observation was in Pontypridd, about 14 miles north of Cardiff, three miles upstream from the previous sampling site on the river Taff.

THE ESTATE

The area chosen was a residential estate housing 4000 persons containing neither industry nor retail butchers shops. Samples of surface draining water were examined with negative results. Pilot observations on the main sewer revealed that specimens were consistently positive for salmonellas. Sampling points were chosen spreading into the branches of the sewerage system; these points were kept very constant throughout the investigation. A sewer plan with the sampling points used is shown in Fig. 1.

MATERIALS

Moore's swabs (Moore, 1948) were used for the survey. The swab technique has been employed routinely for monitoring salmonellosis in our area since 1955 (Harvey & Phillips, 1955; Harvey, 1957; Harvey & Phillips, 1961; Harvey, Price,

Bate & Allen, 1963; Harvey, Price & Dixon, 1966). It has been applied to bakehouses, abattoirs, knackers yards, retail meat markets and wholesale and retail butchers. By its use, information has been collected on sources of infection in sporadic and epidemic salmonellosis, on the average seasonal degree of abattoir contamination (Harvey, 1965), and on the type of domestic animal excreting salmonellas. Such information would not have been so easily gained in other ways. We now wished to discover the incidence of salmonellas in sewage from a population of 4000 people and to isolate all serotypes present (in so far as this was technically possible).

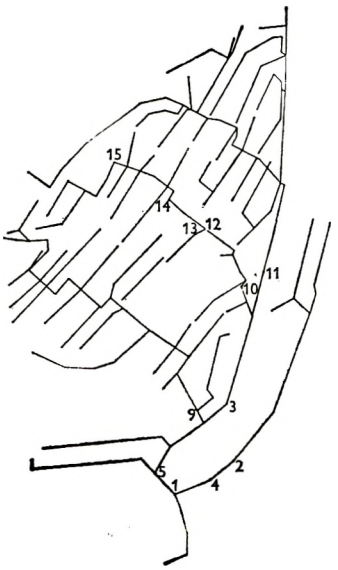
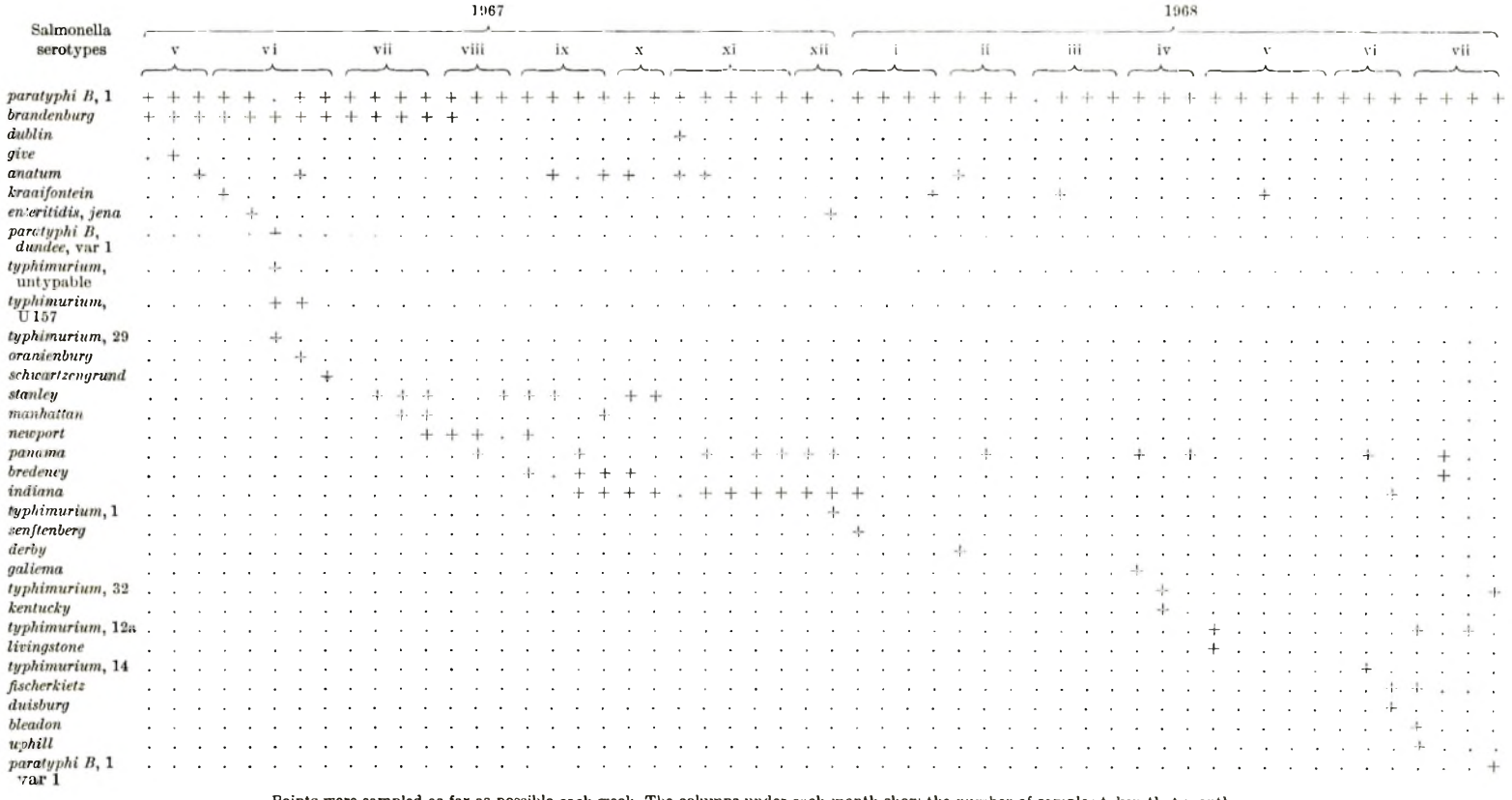


Fig. 1. Sampling points used on sewerage system.

METHODS

The cultural methods used were largely developed in this laboratory. The swabs were brought in weekly, having remained 7 days in the sewers. They were placed, on removal from the sampling point, into 1 lb. screw-capped jars. At the laboratory, selenite F broth was poured into the jars in sufficient volume to cover the swabs. The enrichment broths were incubated at 43° C. for 24 hr. (Harvey & Price, 1968). Subcultures were made to deoxycholate citrate agar and brilliant green MacConkey agar (Harvey, 1956). The plates were incubated at 37° C. and the brilliant green MacConkey plates were examined after 24 hr. incubation. The deoxycholate plates were incubated for 48 hr. to aid differentiation between salmonella and proteus colonies (Harvey & Price, 1968). Suspicious colonies were picked for identification. The deoxycholate citrate agars were used for secondary enrichment by the Craigie tube method of Harvey & Price (1967*b*). All positive samples were finally examined for multiple serotypes by the serological technique of Harvey & Price (1967*a*).

Table 1. *Isolation of different salmonella serotypes from a residential estate in Pontypridd*



Points were sampled as far as possible each week. The columns under each month show the number of samples taken that month.

Table 2. *Salmonella isolations from different sampling points*

Salmonella serotypes	Sampling point 1	2	3	4	5	9	10	11	12	13	14	15	Other isolations in Glamorgan
paratyphi B, 1	46	2	.	.	38	2	2	.	1	.	1	.	
brandenburg	10	2	.	1	8	2	8	1	2	3	1	.	Drains, Central Market, Cardiff, 16. v. 57
dublin	.	.	1	.	.	.	.	.	.	.	.	.	Pig & cattle drains, Cardiff abattoir, 1. v. 67
give	.	.	.	1	.	.	.	.	.	.	.	.	
anatum	3	.	.	.	5	1	2	3	.	.	.	.	Cattle drain, Cardiff abattoir, 18. iv. 67 Knackers yard 24. xi. 67
kraaifontein	.	.	.	.	.	.	1	3	.	.	.	.	
enteritidis, jena	.	.	.	.	1	.	.	1	.	.	.	.	
paratyphi B, dundee, var. 1	1	.	.	.	.	.	.	.	.	.	.	.	
typhimurium, untypable	1	.	.	.	.	.	.	.	.	.	.	.	
typhimurium, U 157	1	1	.	.	.	.	.	1	.	.	.	.	
typhimurium, 29	.	.	.	.	1	.	.	.	.	.	.	.	
oranienburg	.	.	.	.	.	.	.	1	.	.	.	.	
schwarzengrund	.	.	.	.	.	1	.	.	.	.	.	.	
stanley	6	1	.	.	4	.	5	2	.	4	1	.	
manhattan	1	.	.	.	1	.	1	1	.	.	.	.	Drains, Central Market Cardiff, 25. ix. 67
newport	.	1	.	.	1	.	1	3	.	.	.	.	
panama	5	1	.	.	3	.	3	4	2	.	4	1	Drains, Central Market, Cardiff, 14. xi. 67
bredeney	3	.	.	.	3	1	.	4	.	.	.	.	Cardiff abattoir, 8. vii. 68
indiana	4	.	.	.	2	.	2	7	.	.	.	.	Pontypridd abattoir, 26. vii. 67; Cardiff abattoir, 27. xi. 67
typhimurium, 1	1	.	.	.	.	.	.	.	.	.	.	.	
senftenberg	.	.	.	.	.	.	.	1	.	.	.	.	Drains, Central Market, Cardiff, 9. i. 68
derby	1	.	.	.	1	.	.	.	.	.	.	.	
galiema	.	.	.	.	.	.	1	.	1	.	.	.	
typhimurium, 32	1	.	.	.	.	.	1	.	.	.	.	.	
kentucky	.	.	.	.	.	.	.	.	1	.	.	.	
typhimurium, 12a	.	.	.	.	2	.	2	.	.	.	.	1	
livingstone	.	.	.	.	.	.	.	.	.	.	1	.	Pig drains, Pontypridd abattoir, 25. iv. 68; 2. v. 68; 9. v. 68
typhimurium, 14	.	.	.	.	.	.	.	.	.	.	1	.	
fischerkietz	.	.	.	.	2	.	1	.	1	.	1	.	
duisburg	.	.	.	.	.	.	.	.	.	.	1	.	
bleadon	.	.	.	.	.	1	.	.	.	.	.	.	
uphill	.	.	.	.	.	1	1	.	.	.	.	.	
paratyphi B, 1, var. 1	.	.	.	.	.	1	.	.	.	.	.	.	

RESULTS

The results are given in Tables 1 and 2. Table 1 records the serotype isolations in terms of time and Table 2 in terms of sampling point. The tables are self-explanatory.

DISCUSSION

The main object of this investigation was to provide evidence that man is frequently infected with a wide range of salmonella serotypes. The term infection used here does not distinguish between latent and overt salmonellosis and epidemiologically it would be difficult to justify such a distinction. Serotypes producing latent infection in one area may produce, by chance, overt infection in another. We believe that we have succeeded in our object and that salmonellosis has been shown to occur regularly in a population of 4000 persons. We can indeed take this a step further if we consider the information derived from Point 11 (Table 2). The population sampled at this point was estimated at 1000 and no shops of *any* sort were situated in this section of the estate. At this sampling point 48% of specimens were positive for salmonellas. Some serotypes in Table 1 are frequently isolated (*S. stanley*, *S. panama*, *S. indiana*, *S. brandenburg*). These probably represent local 'prevalences' (Parker, 1954). *S. stanley* and *S. panama* have recently caused considerable illness in France as well as in the United Kingdom (Le Minor *et al.* 1967; Vernon, 1967) and the local isolations may well reflect a wider geographical distribution. In this series *S. typhimurium* does not dominate the pattern which contrasts with the usual finding in overt salmonellosis in man. Other serotypes less frequently recorded in Table 1 suggest an exotic origin (*S. kraaifontein*, *S. galiema*, *S. fischerkietz*, *S. duisburg*, *S. bleadon*, *S. uphill*).

While overt salmonella infection was not noted in the estate, some of the serotypes isolated caused clinical infection elsewhere in Glamorgan, e.g. *S. stanley*, *S. panama*, *S. indiana* and *S. fischerkietz*.

From the technical aspect there are some points worth noting. The amount of information recorded in Table 1 would have been scanty if the serological method for separating serotypes had not been used (Harvey & Price, 1967*a*). The nearer the sampling point was to the actual focus of excretion in the sewerage system, the better the chance of an isolation. If we had confined our investigations to Point 1 (draining the whole estate), less than half the total number of serotypes would have been found. Without multiple sampling points, the number of phage-types of *S. typhimurium* discovered would have been greatly reduced. The regular finding of *S. paratyphi B*, phage-type 1, in Points 1 and 5 (Table 2) probably was due to the existence of a chronic carrier nearby. It was noticed that other serotypes were more readily isolated when points above 1 and 5 were examined. In such an investigation the best results are obviously obtained by using as many sampling sites as is conveniently possible.

The relationship between the isolations from human sewage and the isolation of identical serotypes from other local sources is given in Table 2. This table is merely an extension of work published previously (Harvey, 1957; Harvey & Phillips, 1961). The natural inference is that animal and human salmonellosis are closely inter-

related. We are not going to indulge in any speculation in this paper as to the ultimate source of salmonellosis. Neither shall we, at this stage, express an opinion as to vehicles of spread of the disease from animals to man. Further work on those points is obviously necessary.

SUMMARY

In a residential estate of 4000 persons, containing neither industry nor retail butchers shops, salmonellas were regularly found in the sewerage system. They were frequently found in the sewage of a portion of the estate housing 1000 persons. The range of serotypes found was wide and some types suggested an exotic origin. No overt salmonella infection in the estate was reported during the period of survey, although local general practitioners had been previously alerted. Overt infection due to serotypes found in the survey were, however, reported in other areas of Glamorgan. Multiple sampling points in the sewerage system and a serological technique for examining samples contaminated with multiple salmonella serotypes were essential for the technical success of the survey.

We should like to thank Prof. Scott Thomson for his advice in the preparation of this paper; Dr E. S. Anderson of the Central Enteric Reference Laboratory and Bureau, Colindale, for phage-typing the strains of *S. typhimurium* and *S. paratyphi B*; and Dr G. J. G. King of the Public Health Laboratory, Bournemouth, for identifying the serotypes isolated. We should also like to thank Mr T. R. Liddington and Mr J. H. Price for their technical assistance.

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Control of cross-infection in an intensive care unit

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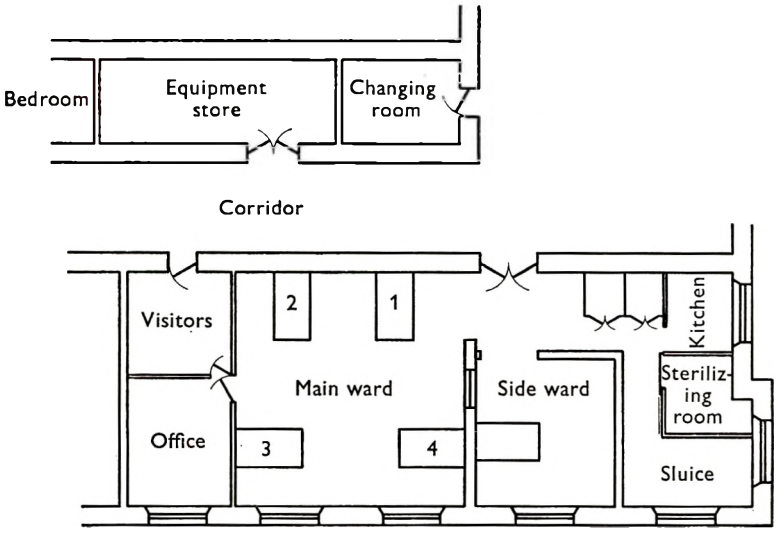
Considerable attention is currently being paid to the problem of controlling cross-infection in intensive care units. Patients requiring endotracheal intubation, tracheostomy or intermittent positive pressure ventilation (I.P.P.V.) are particularly susceptible to infection, often by *Pseudomonas aeruginosa* and organisms of the coliform/*Proteus* group. Many strains of these species are not antibiotic-sensitive, and their accumulation in a unit caring for severely ill patients presents a formidable hazard. This paper describes the routine measures we employ to prevent cross-infection, and records our experience of infections occurring during the first 13 months of our unit's existence.

NATURE OF THE UNIT

The Intensive Therapy Unit cares chiefly for patients in respiratory failure, unconscious patients, and those requiring continuous E.C.G. monitoring for coronary thrombosis and cardiac arrhythmia. The construction of the unit is shown in the figure (the numerals indicate the four beds in the main ward).

Walls and ceilings have smooth, painted surfaces; dust-gathering protuberances are reduced to a minimum, and windows are double-glazed. Air admitted to the



Plan of the Intensive Therapy Unit at the Royal Hospital, Sheffield.

main ward via bacterial filters is maintained at a positive pressure with respect to the corridor; air in the side ward, maintained at a pressure 5 lb./in.^{2} higher than the main ward, finds its outlet through a loaded wall vent into the sluice and thence into the street. Welded-seam vinyl sheet flooring is used throughout the unit.

Most patients requiring I.P.P.V. are nursed on the unit's three Cape ventilators. A Watson 'Barnet' Mk. III ventilator is used for cases requiring a 'square-wave' form of ventilation. Humidification for patients with tracheostomies or endotracheal tubes *in situ* is provided by ultrasonic and cold air nebulizers.

METHODS

Measures designed to prevent cross-infection

After use by a patient, the internal components and exterior of the ventilators are cleaned with 1/200 alcoholic chlorhexidine; sterilization is not routinely attempted. Bacterial filters are not employed in the ventilator circuit, but we attempt to prevent initial contamination of the ventilators by daily autoclaving of the connector tubing. Moistened swabs are used to sample the bacterial flora of the tubing before sterilization. The exhaust of the ventilator, and walls, window-ledges and other surfaces in its vicinity, are sampled during ventilation. Humidifiers are filled with 1/5000 aqueous clorhexidine (Phillips & Spencer, 1965) which is changed daily and tested for sterility weekly. Those not in use are sealed with sterile rubber bungs. Perspex tracheostomy cages are cleaned with alcoholic chlorhexidine then sterilized by boiling, and sterile tracheostomy sets are provided on Bowie trays. Sterile disposable forceps are used for changing tracheostomy dressings and inserting disposable suction catheters. Wall suction tubing is autoclaved daily in the main hospital autoclave; a small autoclave in the unit is used for bowls and loose instruments. Disposable bed-pans and urinals are macerated in a 'Clinimatic' disposal unit. Ceilings and walls are washed regularly, and bed areas are washed with 1% 'Stericol' (Izal Ltd.) after the discharge of grossly infected patients.

If the side ward contains an infected patient, the patient in bed 1 is potentially at greater risk of infection than the other patients when the door of the side ward is opened; bed 1 is therefore now used only when no other is available.

Infections occurring among the staff are investigated, and affected personnel suspended from duty until bacteriological cure is achieved.

Bacteriological methods

Sputum, urine and swabs from tracheostomies, other wounds and infective lesions are examined twice weekly throughout the patient's stay. Conventional methods are employed, except that culture in 10% CO_2 is preferred to aerobic culture for all specimens except urine. Swabs from equipment are cultured on blood agar, incubated aerobically for 4 days. Humidifier fluid is neutralized with Lecithin/Lubrol W broth and incubated at 37° C. for 4 days; the control organism is *Staphylococcus aureus*. Sensitivity tests to eleven antibiotics are performed on 'Oxoid' sensitivity-test agar plates flooded with a broth culture. Sulphonamide

sensitivity tests are performed on antagonistic-factor-free agar containing varying concentrations of sulphonamide; a light inoculum is employed. All strains of *Staph. aureus* are phage-typed (Blair & Williams, 1961).

Each potentially pathogenic species isolated from a specimen was considered to represent one 'infection' (though the term 'infection' is somewhat unsatisfactory, since colonization by potential pathogens did not always affect the patients' clinical course). Some patients had infections with the same species in more than one site; each site was considered a separate 'infection' only if the organisms belonged to demonstrably different strains. In Table 3 such infections are classified by the site in which they first appeared. Repeated isolation of the same strain from one site constituted one infection.

RESULTS

Clinical results

There were 207 admissions, representing 196 patients (nine being admitted on two occasions and one on three occasions). The patients fall into three groups: (I) Those requiring I.P.P.V.; (II) Patients, other than those in group I, requiring endotracheal intubation or tracheostomy; (III) Patients admitted for continuous monitoring. The numbers of patients in each of these three groups, with the numbers admitted from Casualty (i.e., from outside a hospital environment), or from the wards or other hospitals, are shown in Table 1; this also shows the incidence of infection, on admission and acquired in the unit, for each of these groups. It is seen that there is a considerable difference between the incidence of infection on admission in patients admitted from Casualty and that in patients

Table 1. *The incidence of infection among patients admitted from the Casualty Department, or from the wards or other hospitals, in relation to the type of treatment given*

	Total patients		Patients admitted from Casualty			Patients admitted from wards or other hospitals		
	No.	Died	No.	Infected on admission	Infection acquired	No.	Infected on admission	Infection acquired
I	85	44	34	1	10 (29)*	51	15 (29)	24 (47)
II	27	6	12	0	3 (25)	15	5 (33)	6 (40)
III	95	13	48	0	1	47	4 (8)	9 (19)
Total	207	63	94	1	14 (15)	113	24 (21)	39 (34)

Group I. Patients requiring I.P.P.V. Group II. Patients other than those in group I, requiring endotracheal intubation or tracheostomy. Group III. Patients admitted for continuous monitoring. *Figures in parentheses indicate percentages.

transferred from the wards or from other hospitals. Those transferred from a hospital environment also show a greater tendency to acquire infection during their stay in the unit; this difference is not real, because 17 of the 34 patients in group I who were admitted from Casualty were cases of drug overdosage, and were basically healthy before admission. Furthermore, many patients in group I admitted from the wards already had respiratory embarrassment or other

Table 2. *Influence of I.P.P.V. on acquisition of infection*

	Patients requiring I.P.P.V.						Patients requiring tracheostomy or endotracheal tube, but not I.P.P.V.					
				No. with infection						No. with infection		
Diagnosis	Total No.	No. with E.T.T.	No. with T.	On admission	Acquired	Total	Total no.	No. with E.T.T.	No. with T.	On admission	Acquired	Total
Drug overdose	18	18	0	1	3	4	8	8	0	0	0	0
Intracranial haemorrhage, etc.	12	12	0	0	3	3	4	3	1	0	3	3
Respiratory inadequacy and post-op. complications	19	13	6	7	10	17	7	4	3	4	2	9
Totals	49	43	6	8	16	24 (48·7%)	19	15	4	1	6	9 (47·4%)

E.T.T. = Endotracheal tube. T. = Tracheostomy.

conditions which rendered them liable to infection. In groups II and III the proportion of patients admitted on account of primary lung disease or major trauma was much lower than in group I.

Table 2 is an attempt to assess the influence of I.P.P.V. on the acquisition of infection. For the three categories of patient considered (all of whom had been subjected to tracheostomy or endotracheal intubation), the incidence of infection was approximately the same whether I.P.P.V. had been used or not.

Table 3. *Bacteria responsible for infections*

Causative organism	No. of infections	Site of infection			
		Sputum	Trache-ostomy	Wound swab	Urine
Coliforms	39	18	6	5	10
Ps. aeruginosa	21	11	7	2	1
Proteus spp.	9	5	1	1	2
H. influenzae	6	5	1	0	0
Strep. pneumoniae	3	3	0	0	0
Staph. aureus	21	10	4	7	0
Strep. faecalis	4	0	1	3	0
Strep. pyogenes	3	0	0	3	0
Candida spp.	14	3	11	0	0
Clostridium welchii	1	0	0	1	0
Totals	121	55	31	22	13

Bacteriological results

The bacteria responsible for the infections diagnosed during life are classified in Table 3. In addition, two infections were diagnosed bacteriologically at autopsy (one case of aspergillosis of the lung, and one of septicaemia caused by a Group A β-haemolytic streptococcus).

We use the term 'coliform' to include all lactose-fermenting Gram-negative bacilli. Precise biochemical identification and serological typing were not considered feasible as a routine, so the antibiotic sensitivity pattern was used as the epidemiological 'marker' for these strains. Twenty different patterns were detected of which four accounted for 19 of the 39 isolations. Most of the remaining types were isolated only once during the period, and types isolated more than once were widely spaced in their occurrence as a rule. On two occasions the same sensitivity-type was isolated almost simultaneously from two patients. In each case the strains involved were obtained from the patients' sputum immediately after their transfer from other wards; there was no evidence to suggest that cross-infection had occurred within the unit.

Table 3 shows that the incidence of infection with *Ps. aeruginosa* was comparatively low. Infections with this species were distributed evenly throughout the period. Precise information on the possibility of cross-infection is lacking, since pyocine typing was introduced only towards the end of this period, but one episode of probable cross-infection is considered below. Typing is now carried out on all strains, and it is our preliminary impression that little cross-infection occurs.

Six phage-types of *Staph. aureus* were encountered, and six strains were untypable. There was no evidence of cross-infection between patients.

Bacteriological tests on equipment

All samples of humidifier fluid were sterile, adequate neutralization of the chlorhexidine being demonstrated by profuse growth of the control organism.

Ventilator tubing was sampled on 68 occasions. The tracheal end and tracheostomy cage or Y-piece invariably yielded bacteria identical with those in the patient's sputum; organisms were isolated on six occasions from the distal end of the expiratory tubing, but never from any point further on in the expiratory circuit, and rarely from the inspiratory tubing. Three cases are of particular interest:

Patient A, a case of terminal true emphysema, was nursed on the same ventilator for 4 weeks, during which *Ps. aeruginosa*, *Staph. aureus* and a coliform organism were repeatedly isolated from sputum, tracheostomy wound and the proximal expiratory tubing, and on four occasions from the distal end of the expiratory tubing. These organisms were never isolated from the exhaust of the machine or from surfaces in its vicinity. After A's death, the ventilator was dismantled as far as possible. The bellows were found to be dry and clean internally; swabs taken from the interior of the bellows, and from various points in the internal circuit, proved sterile on culture.

Patient B was admitted in coma 3 days before A's death, into bed 2 (A was in bed 3). I.P.P.V. was begun with a ventilator which had not been used recently, but stored in a clean, dry condition. *Ps. aeruginosa* was soon isolated from the sputum and proximal and distal ends of both inspiratory and expiratory tubing, but not from the exhaust or the humidifier fluid. Patient B died 1 week after admission; bacteriological tests on the ventilator after his death yielded entirely negative findings.

Patient C, in acute renal failure, was then being nursed in the side ward on the ventilator just vacated by A, and her sputum became infected by *Ps. aeruginosa* of the same pyocine type as that isolated from B. After C's death, the internal parts of her ventilator were again found to be sterile. The strain of *Ps. aeruginosa* isolated from A was unfortunately not typed; it seems likely that A was the source of the infection in B and C, but it is impossible to conclude that the ventilators were concerned in its transmission.

DISCUSSION

It is difficult to compare the incidence of infection in our unit with that seen in other centres, since there is a striking lack of published information on this point. However, we consider our experience to be unusual in three respects. First, the majority of 'infections' in our unit have been associated with coliform bacilli rather than with *Ps. aeruginosa* or *Staph. aureus*. Secondly, we have not been able to demonstrate much evidence of cross-infection. Finally, in the very few cases where cross-infection may have occurred, we have never been able to incriminate the ventilators in its transmission.

Cross-infection has been a major problem in other units (see Campbell, Reid, Telfer & Fitch, 1967; Tinne, Gordon, Bairn & Mackey, 1967), though its incidence has not been clearly defined. The causative organism in most units has been *Ps. aeruginosa*. Where ventilators and suction equipment have acted as vectors of infection, the published reports either demonstrate grave deficiencies in basic hygiene (e.g., Sutter, Hurst, Grossmann & Calonje, 1966) or give no details of routine preventive measures before the outbreak (Tinne *et al.* 1967).

Various attempts have been made to sterilize ventilators after use, by means of ethylene oxide fumigation (Bishop, Robertson & Williams, 1964) or antibacterial aerosols (Judd *et al.* 1968; Meadows, Richardson, Fish & Williams, 1968; Spencer, Ridley, Eykyn & Achong, 1968). Other writers recommend prevention of contamination by means of bacterial filters; this seems a logical approach, but existing filters are not ideal.

Our frequent changing and autoclaving of connector-tubing prevents multiplication of Gram-negative bacilli, since moisture does not accumulate and dry conditions are unfavourable to these bacteria (Lowbury & Fox, 1953; Pettit & Lowbury, 1968). There is then insufficient accumulation of bacteria in the tubing to make contamination of the ventilator likely. The use of aqueous chlorhexidine for humidification has effectively prevented contamination of the humidifiers from retrograde spread along the inspiratory tubing. We consider that these methods have virtually excluded the possibility of cross-infection occurring via the ventilators in our unit. The main factor predisposing to respiratory infection in our patients appears to be the presence of an endotracheal tube or tracheostomy (especially the latter), and I.P.P.V. does not significantly alter its incidence.

The nature of the basic reservoir of infection is uncertain. Tinne *et al.* (1967), and Rountree & Beard (1968), emphasize the importance of environmental contamination by infected secretions. The patient with an infected tracheostomy is undoubtedly a particularly potent disseminator of organisms; however it is questionable whether their survival in the environment could be sufficient to account for the rather unusual situation found in this unit. The wide variety of coliform strains which we have encountered, and the sporadic nature of the infections, suggest that these infections may be endogenous, the bacteria being derived from the patient's own gut or upper respiratory tract.

It is not quite so easy to explain infections caused by *Ps. aeruginosa*. Most authors have not been able to demonstrate a high faecal carriage rate for this species, though Shooter *et al.* (1966) reported a carrier rate of 24 % in patients admitted to a surgical ward. They noted that isolation was particularly frequent in patients with colostomies, and also that the organism was more commonly found in patients who had previously been exposed to various influences inherent in the hospital environment. It is likely that patients admitted to an intensive care unit might show a similarly increased carrier rate. Certainly the infrequency of this species in our unit and our inability in most cases to demonstrate any means of transmission between patients, suggest that the possibility of endogenous infection is worth investigating.

These problems can only be resolved by a more detailed knowledge of patients'

intestinal bacterial flora and the extent to which these bacteria can contaminate the environment. Investigations into these aspects are now in progress, but meanwhile we feel that the measures described in this report can successfully limit the incidence and spread of infection among these highly susceptible patients.

SUMMARY

In a survey undertaken in an intensive care unit, coliform bacilli were found to be responsible for most infections, *Pseudomonas aeruginosa* and *Staphylococcus aureus* being isolated much less frequently. Tracheostomy or endotracheal intubation predisposed to infection, but in our experience intermittent positive pressure ventilation did not significantly affect its incidence. Little cross-infection has occurred, and it has never been possible to incriminate the ventilators in its transmission.

We gratefully acknowledge the assistance we have received from colleagues in the Intensive Therapy Unit and the Department of Bacteriology during the course of this investigation.

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Tables of the upper limit to the estimate of the density of contaminating particles in a medium

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The expression 'contaminating particles' is used in the broadest sense: micro-organisms, virus particles and mutants of a parent organism are examples from microbiology, but other sciences face a problem similar to that dealt with in this paper. According to the case, the 'medium' can be air, a culture medium, a suspension or any other material containing 'foreign' bodies. It is assumed that the contaminating particles are randomly distributed throughout the medium (i.e. that the probability of finding any individual particle in any one unit volume is constant and that the sampling procedure itself does not interfere with this assumption).

Theoretically, the number of particles found in a random sample is an unbiased estimate of the density of the contaminant in the medium; but this is of little practical value, particularly where the sample drawn proves to be free from contaminants. The determination of an upper limit to the estimate of the density is often required and this upper limit is defined by a probability level previously fixed by the investigator. If, for instance, the 5% upper limit of contamination of a medium is found to be six particles per litre (this is usually called the 5% fiducial or confidence upper limit) the investigator concludes that the density of contamination is not higher, unless he is the victim of a 1 in 20 mischance of sampling. (Obviously the upper limit caters for sampling fluctuations only and the assumption of random distribution must hold good.)

Four cases emerge and will be treated separately: (A) an uncontaminated sample drawn from an infinitely large medium; (B) an uncontaminated sample drawn from a medium of finite size; (C) a contaminated sample drawn from an infinitely large medium; (D) a contaminated sample drawn from a medium of finite size.

(A) *An uncontaminated sample drawn from an infinitely large medium*

'Infinitely large' in this context means that the sample volume is negligible compared with the volume of the medium, e.g. samples of sterilized air. The numbers of contaminating particles in the separate units of volume will follow the Poisson distribution and therefore the upper limit n of its expectation is given by

$$n = -\log_e P = (-\log_{10} P) \times 2{\cdot}3026, \tag{1}$$

where P stands for the selected probability level. This gives for instance $n = 3$ for $P = 5\%$ and $n = 4{\cdot}6$ for $P = 1\%$.

It follows that the question 'How large (assuming it will turn out to be sterile)

must the sample volume v be to ensure a density of not more than n contaminants, the risk of error being P?' is answered by

$$v = \frac{-\log_e P}{n}. \tag{2}$$

Examples

To test its efficiency, a sample of 100 ft.^{3} of effluent air from an air sterilization plant was examined and no contaminants were found. Using (1) it can be concluded that not more than three contaminants per 100 ft.^{3} (on average) will in future defy the sterilization operation, where the probability of being misled by the sample is 5 %, (assuming, naturally, that the conditions of running the plant remain constant). If the test sample were increased to 10^4 or even 10^6 ft.^{3} and still no contaminants were found, this would only show that the upper limit of density of contamination had fallen to three particles per 10^4 or three particles per 10^6 ft.^{3} respectively. Obviously 100 % efficiency cannot be proved by mere sampling.

The same logic applies to a wide variety of cases: e.g. drugs intended for humans are tested with guinea-pigs or a number of flights carried out to demonstrate the safety of an aircraft; if 1000 animals have shown no ill effect or if 1000 sorties have been flown without accident, then the 5 % upper limit is three adverse results per 1000 and no increase in sample size will ever support a claim of absolute safety.

If, however, the air sterilization plant (see above) had been improved so that, when tested, it produced an uncontaminated sample of 100 ft.^{3}, and if it had formerly returned on average five contaminants per 100 ft.^{3}, this would be good evidence for the superiority of the new technique, because the probability of obtaining an uncontaminated sample if the expectation was 5 particles would be small. [Using (1): $P = e^{-5} = 0{\cdot}7$ %.]

(B) *An uncontaminated sample drawn from a medium of finite size*

Let V be the volume of the medium, let n be the maximum acceptable number of contaminating particles per f units of volume, let P be the probability that n is in fact exceeded, let p be the fraction of V to be sampled, let q be equal to $(1-p)$, let Z be equal to $q \log q$, then, provided the sample is found to be uncontaminated,

$$q^{Vqn/f} = P, \tag{3}$$

i.e.
$$q \log q = \frac{f \log P}{Vn} = Z. \tag{4}$$

This equation was solved with the help of a computer for values of Z from $-0{\cdot}004$ ($0{\cdot}001$) to $-0{\cdot}15$, which correspond to p values in the range 0·9–59·9 %. Table 1 records these pairs of values.

Examples

(*a*) How large a sample must be drawn from a culture vessel of 4 l. to state that the rest of the vessel does not contain more than three infective units (of a certain virus) per l., assuming that the sample will be sterile and that the risk of being led into error by the sample is not greater than 5 %?

Here: $V = 4$, $n = 3$, $P = 0{\cdot}05$ ($\log P = -1{\cdot}3$), $f = 1$. Therefore [using (4)] $Z = 1 \times (-1{\cdot}3)/(4 \times 3) = -0{\cdot}1083$ and entering Table 1 at this value for Z gives 29·9 %.

Answer: 29·9 %; that is, 1·2 l. must be sampled.

Table 1. *Percentage of the medium to be sampled*

Z	0	1	2	3	4	5	6	7	8	9
−0·00					0·9	1·2	1·4	1·6	1·9	2·1
1	2·3	2·6	2·8	3·0	3·3	3·5	3·8	4·0	4·2	4·5
2	4·7	5·0	5·2	5·4	5·7	5·9	6·2	6·4	6·7	6·9
3	7·2	7·4	7·7	7·9	8·2	8·4	8·7	8·9	9·2	9·4
4	9·7	10·0	10·2	10·5	10·7	11·0	11·2	11·5	11·8	12·0
−0·05	12·3	12·6	12·8	13·1	13·4	13·6	13·9	14·2	14·5	14·7
6	15·0	15·3	15·6	15·8	16·1	16·4	16·7	17·0	17·2	17·5
7	17·8	18·1	18·4	18·7	19·0	19·3	19·5	19·8	20·1	20·4
8	20·7	21·0	21·3	21·6	21·9	22·3	22·6	22·9	23·2	23·5
9	23·8	24·1	24·5	24·8	25·1	25·4	25·7	26·1	26·4	26·7
−0·10	27·1	27·4	27·8	28·1	28·4	28·8	29·1	29·5	29·8	30·2
1	30·6	30·9	31·3	31·7	32·0	32·4	32·8	33·2	33·6	34·0
2	34·4	34·8	35·2	35·6	36·0	36·4	36·8	37·3	37·7	38·1
3	38·6	39·0	39·5	39·9	40·4	40·9	41·4	41·9	42·4	42·9
4	43·5	44·0	44·5	45·1	45·7	46·3	46·9	47·6	48·2	48·9
−0·15	49·6	50·4	51·2	52·0	52·9	53·9	54·9	56·2	57·6	59·5

(*b*) If in the previous example the acceptable upper limit was six infective units per 100 ml. and the vessel contained 30 l.: $V = 30$, $n = 6$, $P = 0{\cdot}05$, $f = 0{\cdot}1$; then $Z = -0{\cdot}0007$; this Z value is smaller than the table provides for and a Poisson distribution is a fair approximation, formula (2) supplying the answer. [3/6 unit volumes $= 0{\cdot}5 \times f = 0{\cdot}05$ l.]

(*c*) A sample of 0·5 l. from a 4 l. vessel was found to be uncontaminated; 1 % fiducial upper limit to the density in the remaining part of the culture is required. Starting from the sample size which is 12·5 % (and taking as unit volume 100 ml.) Table 1 gives the corresponding Z value of −0·0506 and changing the subject of formula (4):

$$n = \frac{f \log P}{VZ} = 0{\cdot}1 \times (-2)/[4 \times (-0{\cdot}0506)] = 0{\cdot}99.$$

Answer: There should not be more than one infective unit per 100 ml. in the vessel unless there has been a one in hundred mischance in sampling.

(*d*) If in the previous example the question had been to find the probability that the unknown density should not be more than 0·5 per 100 ml. the subject of (4) is again changed:

$$\log P = \frac{ZnV}{f} = -0{\cdot}0506 \times 0{\cdot}5 \times 4/0{\cdot}1 = -1{\cdot}0133 = \bar{2}{\cdot}9867 \text{ and } P = 0{\cdot}09699.$$

Answer: The probability of the density exceeding 0·5 infective units per 100 ml. is approx. 9·7 %.

(C) *A contaminated sample drawn from an infinitely large medium*

In most cases the investigator trying to assess the density of contaminants expects only a small number of them and hopes to find none in his sample. If, however, the sample turns out to be contaminated he might want to assign an upper limit to the most probable density determined by k, the number of contaminants observed in the sample. The problem is to find the expectation n of a Poisson distribution so that the chance of obtaining a sample containing k or less is equal to the desired probability level or, in mathematical form, the solution for n of the equation

$$\sum_{k=0}^{k} \frac{e^{-n} k^n}{k!} = P. \tag{5}$$

This equation can be solved by trial and error (for instance), preferably with the help of a computer. Table 2 may prove useful. (The figures are given to the nearest first place of decimals that would keep P below the quoted value.) Other tables exist giving confidence or fiducial limits for the expectation of the Poisson distribution, e. g. Fisher & Yates, *Statistical Tables*, table VIII, 1, and *Biometrika Tables*, table 40.

Table 2. *The probability* P *that the density in an infinitely large medium will exceed the indicated number, if* k *particles are found in the sample*

k ...	1	2	3	4	5	6	7	8	9	10
10 % P	3·9	5·4	6·7	8·0	9·3	10·6	11·8	13·0	14·3	15·5
5 % P	4·8	6·3	7·8	9·2	10·6	11·9	13·2	14·5	15·8	17·0
1 % P	6·7	8·5	10·1	11·7	13·2	14·6	16·1	17·5	18·8	20·2
0·1 % P	9·3	11·3	13·1	14·9	16·5	18·1	19·7	21·2	22·7	24·2
0·01 % P	11·9	14·0	16·0	17·9	19·7	21·4	23·1	24·7	26·3	27·9

Example

A yearly average of 12 cases of a disease used to be reported in a certain country. After measures claiming to be of prophylactic value had been introduced the incidence dropped in the first year to two cases. Is this convincing evidence for the efficacy of these measures? Since it seems reasonable to assume a Poisson distribution, Table 2 is entered at $k = 2$ and 12·0 is found to lie between $P = 0{\cdot}1$ % and 0·01 %, which makes it extremely unlikely that the drop in the number of cases is the result of mere sampling fluctuation. Answer: Yes (naturally provided that any other cause of the decrease in incidence of the disease can be ruled out).

(D) *A contaminated sample drawn from a medium of finite size*

Let p be the proportion of the medium to be sampled, let q be equal to $(1-p)$, let k be the number of contaminating particles observed in the sample, let N be the upper limit of the number of particles in the remaining medium, let P be the probability level defining the upper limit, let m be equal to $(N+k)$, then equating the tail of the appropriate binomial distribution to P:

$$\sum_{r=0}^{k} q^{m-r} p^r = P, \tag{6}$$

Table 3. 5 % (1%) *fiducial upper limit to the estimate of the remaining number of contaminating particles in the medium after a sample of* p % *of it contained* k *particles*

k...	0	1	2	3	4	5	6	7	8	9	10
p											
1	299 (459)	472 (661)	626 (836)	770 (999)	909 (1153)	1044 (1302)	1176 (1447)	1305 (1589)	1433 (1728)	1559 (1965)	1683 (2000)
2	149 (228)	235 (329)	311 (416)	383 (496)	452 (573)	518 (647)	584 (719)	648 (789)	711 (858)	773 (926)	835 (993)
3	99 (152)	156 (218)	206 (275)	254 (329)	299 (380)	343 (428)	386 (476)	429 (522)	470 (568)	512 (613)	553 (657)
4	74 (113)	116 (163)	154 (205)	189 (245)	223 (283)	256 (319)	288 (354)	319 (389)	350 (423)	381 (456)	411 (489)
5	59 (90)	92 (129)	122 (163)	150 (195)	177 (225)	203 (254)	228 (282)	253 (309)	278 (336)	302 (362)	326 (388)
6	49 (75)	79 (107)	101 (135)	124 (161)	146 (186)	168 (210)	189 (233)	210 (256)	230 (278)	250 (300)	270 (321)
7	42 (64)	65 (91)	86 (115)	106 (137)	125 (158)	143 (179)	161 (198)	178 (218)	195 (236)	212 (255)	230 (273)
8	36 (56)	57 (80)	75 (100)	92 (119)	108 (138)	124 (155)	140 (172)	155 (189)	170 (205)	184 (221)	199 (237)
9	32 (49)	50 (70)	66 (89)	81 (106)	96 (122)	110 (137)	123 (152)	136 (167)	150 (181)	173 (195)	176 (209)
10	29 (44)	45 (63)	59 (79)	73 (94)	85 (109)	98 (122)	110 (136)	122 (149)	134 (162)	145 (174)	157 (187)
11	26 (40)	41 (57)	54 (72)	66 (85)	77 (98)	88 (110)	99 (123)	110 (134)	120 (146)	131 (157)	141 (169)
12	24 (37)	37 (52)	49 (66)	60 (78)	70 (90)	80 (101)	90 (112)	100 (122)	109 (133)	119 (143)	128 (153)
13	22 (34)	34 (48)	45 (60)	55 (71)	64 (82)	74 (92)	83 (102)	91 (112)	100 (122)	109 (131)	117 (140)
14	20 (31)	31 (44)	41 (55)	50 (66)	59 (75)	68 (85)	76 (94)	84 (103)	92 (112)	100 (121)	108 (129)
15	19 (29)	29 (41)	38 (51)	47 (61)	55 (70)	63 (79)	70 (87)	78 (96)	85 (104)	93 (112)	100 (120)
16	18 (27)	27 (38)	36 (48)	44 (57)	51 (65)	58 (73)	65 (81)	72 (89)	79 (96)	86 (104)	93 (111)
17	17 (25)	25 (36)	33 (45)	41 (53)	48 (61)	54 (68)	61 (76)	68 (83)	74 (90)	80 (97)	87 (104)
18	16 (24)	24 (33)	31 (42)	38 (50)	45 (57)	51 (64)	57 (71)	63 (78)	69 (84)	75 (91)	81 (97)
19	15 (22)	23 (32)	29 (39)	36 (47)	42 (54)	48 (60)	54 (67)	60 (73)	65 (79)	71 (85)	76 (91)
20	14 (21)	21 (30)	28 (37)	34 (44)	40 (51)	45 (57)	51 (63)	56 (69)	61 (75)	67 (80)	72 (86)
21	13 (20)	20 (28)	26 (35)	32 (42)	37 (48)	43 (54)	48 (59)	53 (65)	58 (70)	63 (76)	68 (81)
22	13 (19)	19 (27)	25 (33)	30 (39)	35 (45)	40 (51)	45 (56)	50 (62)	55 (67)	59 (72)	64 (77)
23	12 (18)	18 (25)	24 (32)	29 (37)	34 (43)	38 (48)	43 (53)	47 (58)	52 (63)	56 (68)	61 (73)
24	11 (17)	17 (24)	22 (30)	27 (36)	32 (41)	36 (46)	41 (51)	45 (55)	49 (60)	53 (65)	58 (69)
25	11 (17)	17 (23)	21 (29)	26 (34)	30 (39)	35 (44)	39 (48)	43 (53)	47 (57)	51 (61)	55 (66)
26	10 (16)	16 (22)	20 (27)	25 (32)	29 (37)	33 (42)	37 (46)	41 (50)	45 (54)	48 (59)	52 (63)
27	10 (15)	15 (21)	20 (26)	24 (31)	28 (35)	32 (40)	35 (44)	39 (48)	43 (52)	46 (56)	50 (60)
28	10 (15)	14 (20)	19 (25)	23 (30)	26 (34)	30 (38)	34 (42)	37 (46)	41 (50)	44 (53)	47 (57)
29	9 (14)	14 (19)	18 (24)	22 (28)	25 (32)	29 (36)	32 (40)	36 (44)	39 (47)	42 (51)	45 (55)
30	9 (13)	13 (19)	17 (23)	21 (27)	24 (31)	28 (35)	31 (38)	34 (42)	37 (45)	40 (49)	43 (52)
31	9 (13)	13 (18)	17 (22)	20 (26)	23 (30)	26 (33)	30 (37)	33 (40)	36 (44)	39 (47)	41 (50)
32	8 (12)	12 (17)	16 (21)	19 (25)	22 (29)	25 (32)	28 (35)	31 (39)	34 (42)	37 (45)	40 (48)
33	8 (12)	12 (17)	15 (21)	18 (24)	21 (28)	24 (31)	27 (34)	30 (37)	33 (40)	35 (43)	38 (46)
34	8 (12)	11 (16)	15 (20)	18 (23)	21 (27)	23 (30)	26 (33)	29 (36)	31 (39)	34 (41)	37 (44)
35	7 (11)	11 (15)	14 (19)	17 (22)	20 (26)	23 (29)	25 (31)	28 (34)	30 (37)	33 (40)	35 (43)
36	7 (11)	11 (15)	14 (18)	16 (22)	19 (25)	22 (28)	24 (30)	27 (33)	29 (36)	31 (38)	34 (41)
37	7 (10)	10 (14)	13 (18)	16 (21)	18 (24)	21 (27)	23 (29)	26 (32)	28 (34)	30 (37)	33 (39)
38	7 (10)	10 (14)	13 (17)	15 (20)	18 (23)	20 (26)	22 (28)	25 (31)	27 (33)	29 (36)	31 (38)
39	7 (10)	10 (13)	12 (17)	15 (19)	17 (22)	19 (25)	22 (27)	24 (30)	26 (32)	28 (34)	30 (37)
40	6 (10)	9 (13)	12 (16)	14 (19)	17 (21)	19 (24)	21 (26)	23 (29)	25 (31)	27 (33)	29 (35)

and solving for m (by computer) establishes the N values in Table 3 for $P = 5\%$ and $P = 1\%$ [m being the nearest integer not to exceed P].

Example

Taking the data of example (*a*) page 534: If the sample of 29·9 % of the vessel had returned a count of two infective units, Table 3 should have been consulted at $p = 30$ and $k = 2$, giving N (the 5 % fiducial upper limit of infective units in the remaining 2·8 l. of the culture) as 17, amounting to an upper limit of density of 6·1 infective units per litre instead of the density of three which had been fixed before the sample was taken, assuming it would turn out to be uncontaminated.

Table 3 can readily be extended to any value of P and k using the normal as an approximation to the binomial distribution, involving the solution of the following quadratic equation in m;

$$m^2p^2 - m(2pq + t^2pq) + k^2 = 0, \tag{7}$$

where t stands for the normal equivalent deviate of P (bearing in mind that P refers to a single tail and the numerically greater solution applies).

The authors hope that the present paper, particularly the tables, will prove to be useful in many fields besides microbiology.

SUMMARY

The theory of assigning an upper limit to the estimate of the degree of contamination of a medium is briefly explained. Tables to save computational labour are presented and their use elucidated by examples.

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Antigenic analysis of vibrio culture filtrate and vaccine El Tor vibrio

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INTRODUCTION

In the course of an investigation on live oral cholera vaccine, Bhattacharya, Narayanaswami & Mukerjee (1968) found that intra-intestinal administration of an El Tor (water) strain, ME7, produced antitoxic immunity in the intestinal tract of the adult rabbit. This suggested that although water strains of *Vibrio eltor* are not known to be toxinogenic, they possibly produce choleragenic toxin in quantities too low to be detected in presently available experimental models. Since the choleragenic toxin (toxin type 2 of Burrows (1968) classification) is known to be heat-labile and antigenically active, the present study was therefore undertaken with a view to examining whether El Tor (water) strains contain the same heat-labile antigens as those of *Vibrio cholerae* and El Tor (case) strains by means of gel diffusion and intra-gel absorption tests using an antitoxic serum raised against a culture filtrate of the 569B strain of *V. cholerae*.

Another object of the present work was to carry out an antigenic analysis of the usual toxin preparation consisting of a dialysed culture filtrate of 569B *V. cholerae*. Finkelstein, Atthasampunna, Chulasamaya & Charunmethee (1966) and Kasai & Burrows (1966) have also reported that the toxin is antigenic and stimulates the production of serum antibodies in rabbits which are capable of neutralizing choleragenic activity. Kasai & Burrows (1967) subsequently demonstrated that heat-denatured toxoid remains fully immunogenic.

It is of interest to note that both Finkelstein and Burrows used partially purified preparations of toxin in their work. Perhaps it was for this reason that Finkelstein & Atthasampunna (1967) administered the toxin with Freund's adjuvant in order to elicit protective immunity against subsequent challenge. In our laboratories we have observed that preparation of the crude toxin induces protective immunity against challenge with cholera toxin and with live vibrios in adult rabbit ileal loops only if administered along with O antigen in the form of heat-killed vibrios (S. C. Sanyal, A. Narayanaswami and S. Mukerjee, unpublished work). It would appear, therefore, that there is no advantage in using a purified toxin preparation rather than the unprocessed culture filtrate for the purpose of immunization; in fact the unpurified preparation, because of the presence of O antigen as a contaminant, may be more effectively immunogenic. The present investigation was therefore also designed to provide information about the antigenic constituents of culture filtrates in relation to their usefulness for antitoxic immunization.

MATERIALS AND METHODS

Vibrio strains

Vibrio cholerae strain no. 569B which has been widely used for production of toxin, a known toxinogenic El Tor (case) strain no. GS-9 and an El Tor (water) strain no. W 6 were used. It is proposed to use strain no. W 6 in the development of a live cholera vaccine. This strain has been found to be apathogenic in ligated intestinal loops and infant rabbit models of experimental cholera and also in human volunteers (Bhattacharya & Mukerjee, 1968; Mukerjee, 1963; Mukerjee & Sanyal, 1967). It is apparently non-toxinogenic as the filtrate prepared from it did not give rise to gut-inflammatory reactions in ligated ileal loops of adult rabbits (unpublished records) or in infant rabbits (R. A. Finkelstein, personal communication).

Preparation of cholera toxin

This was prepared from strain 569B according to the method reported by Craig (1966). The toxin in the culture filtrate was precipitated with 70% ammonium sulphate and filtered through a Buchner funnel with Whatman no. 1 filter paper. The residue was dissolved in distilled water and dialysed in the cold against distilled water with four to five changes. It was filtered through a Millipore filter and stored in the refrigerator at 4° C. The preparation will be referred to as 569B toxin. The protein content of this toxin was determined by the method of Lowry, Rosenbrough, Farr & Randall (1951) and adjusted to 1 mg./ml. with distilled water. The potency of the toxin was tested in adult rabbit ileal loops at regular intervals during the whole course of experiment. The minimum dose required for causing definite reaction in a 10 cm. loop was a volume containing 120 μg. protein and this potency remained unchanged throughout the course of the investigation.

Preparation of whole cell lysate

Overnight growth of vibrios in a Roux-flask containing papain agar medium was harvested in 10 ml. of physiological saline and centrifuged. The sediment was washed twice with saline. Finally the sediment was suspended in distilled water to obtain a concentration of 10^{11} vibrios/ml. and sonicated for 20 min. in the M.S.E. Ultrasonic Disintegrater at a frequency of 20 kc./s. The sonicated material was centrifuged in the cold. The supernatant was freeze-dried and stored at 4° C.

Preparation of live and heat-denatured antigens

Overnight growth of vibrios in a Roux flask containing papain agar medium was harvested in 10 ml. of physiological saline and centrifuged. The sediment was washed and resuspended in 10 ml. of saline. Five ml. of this was saved as live antigen and the remaining portion was heated in a boiling water bath for 1 hr. and used as heat-denatured antigen.

Preparation of antitoxic sera

The antitoxic sera were produced in adult rabbits by six injections of graded doses of toxin from 0·25 to 3 ml.; the first two doses were given subcutaneously followed by two intraperitoneal and two intravenous injections at intervals of 4 days. Blood samples were drawn 7 days after the last injection. The sera were stored in the refrigerator at 4° C.

Absorption of sera

The absorption of sera was carried out with heat-denatured and packed sediment of live vibrios. The suspensions were kept in the incubator for 2 hr. with repeated shaking and then overnight in the refrigerator. The sera were collected after centrifugation.

Serological analysis by gel-diffusion and intra-gel absorption tests

Diffusion of antigen and antibodies was carried out according to the method described by Feinberg (1958). The medium contained 0·8% Difco agar. Intra-gel absorption with soluble antigens was carried out to unmask the specific bands. The soluble antigens were mixed with equal volumes of medium and plated. Wells were cut in the medium after solidification. The precipitation bands appeared between the antigen and antibody wells on standing at room temperature in a moist chamber within 3–7 days.

Antigenic analysis by immuno-electrophoresis

The immuno-electrophoretic analysis of 569B toxin was carried out in the LKB apparatus 3290B on microscope slides using 0·8% Ionagar no. 2 and 0·87% sodium azide in the gel medium. Barbital buffer of pH 8·2 was used.

RESULTS

Plate 1, fig. 1, shows that the antitoxic serum produced four precipitin bands against the crude toxic filtrate in well 1, and nine bands against whole cell lysate of 569B strain in well 5. Bands C and D are likely to be due to the heat-labile specific antigens present in the toxin preparation, as they are absent against well 6 which contained heated toxin. Fewer precipitin bands were formed against well 4 where live cells of 569B strain was added. It is likely that although the antigens were present in the intact cells they could not all diffuse into the agar-gel in the absence of lysis. The polysaccharide preparation in well 2 had been prepared from a *V. cholerae* (Ogawa) strain by the method of Shrivastava & Seal (1937). It consisted therefore of the polysaccharide moiety of the heat-stable lipopolysaccharide antigens of wells 1 and 3. On intra-gel absorption with 569B toxin, or whole cell lysate of 569B all precipitin bands due to heat-stable as well as heat-labile antigens in the toxin disappeared.

The El Tor (water) strain no. W6 which is apparently non-toxinogenic but known to have the same antigenic make-up as *V. cholerae* and choleragenic El Tor strains (Mukerjee, 1963) was used for absorption of the antitoxic serum. When this

absorbed serum was used for gel-precipitation with the toxin, whole cell lysates of 569B, whole cell lysates of W6 and live and heat-denatured antigens of 569B and live antigens of GS-9 not a single precipitin band appeared. On the other hand when heat-denatured W6 was used for absorption of the antitoxic serum two continuous precipitin bands appeared against the toxin, whole cell lysates of 569B and of W6, and live GS-9. These bands therefore appear to be due to the heat-labile antigens in the toxin preparation Pl. 1 fig. 2 corresponding to C and D of Pl. 1 fig. 1. It appears therefore that the W6 strain contains the heat labile antigens of cholera toxin although the presence of choleragenic toxin cannot be demonstrated in culture filtrates using the adult or infant rabbit models. The presence of these heat-labile antigens in W6 is further confirmed by the results presented in Pl. 1. fig. 3 where the two bands due to the cholera toxin are seen to be continuous against wells 1, 2, 3 and 6 containing respectively 569B toxin, 569B whole cell lysate, GS-9 live antigen and W6 whole cell lysate.

Immuno-electrophoresis of cholera toxin against cholera antitoxic serum also points to the presence of two specific antigenic constituents in the toxin. The pattern of precipitation on immuno-electrophoresis of cholera toxin is shown in Pl. 1. fig. 4, and is similar to that against well 1 in Pl. 1, fig. 1. Of the four precipitin bands, those near the well may be due to the lipopolysaccharide components present in the crude toxic filtrate as a contaminant which gave bands in corresponding positions in the gel-diffusion experiment. The remaining two precipitin bands may similarly correspond to the specific antigens C and D of Pl. 1, fig. 1.

DISCUSSION

Misra & Shrivastava (1959) using ultrasonic lysates of cholera vibrios have reported that at least seven precipitin bands were formed when tested against cholera antiserum. Of these, the band near the antigen-well he named the α-band and considered it to be due to a lipopolysaccharide-protein fraction of the cell wall. The same number of precipitin bands or even more have also been obtained in the present study when whole cell lysate was titrated against serum raised in rabbits against cholera toxin. This indicates that in the toxic filtrate practically all the antigenic constituents of the vibrio cell were present, including the specific antigens due to cholera toxin.

There is now considerable evidence that the choleragenic toxin, liberated into the culture medium under suitable conditions of culture, is a heat-labile, non-dialysable compound. Coleman *et al.* (1968) have shown that purification of this component ultimately yields two lipoprotein compounds separable by fractionation on DEAE-sephadex, which are antigenically distinct. The present results confirm the presence of two distinct heat-labile antigenic constituents in the toxin, corresponding to the precipitin bands (C, D) shown in Pl. 1, fig, 1. These bands were totally absent when heat-denatured cholera toxin was used against the antitoxic serum. They were found to be completely absorbed in the intra-gel absorption test with cholera toxin.

Disappearance of the precipitin bands due to the strains 569B and GS-9 on

absorption of the serum with live El Tor W 6 but not with heat-denatured suspensions indicates that the heat-labile antigenic fractions are common to these strains of vibrios. Heat treatment denatures the labile protein antigens and the heat-treated suspensions are consequently incapable of absorbing the bands due to toxin, while their capacity to absorb precipitin bands due to heat stable fractions remains unaffected. The gel-precipitation and intra-gel absorption tests thus furnish clear proof that the toxin is present in W 6 even if in concentrations too low to produce pathogenic effects in the rabbit models. The difference between the 569 B, GS-9 and W 6 strains in their capacity to produce toxin appears therefore to be only a quantitative one.

Mukerjee (1963) showed that the apathogenic El Tor (water) strain W 6 is composed of smooth specific somatic antigens identical with those of *V. cholerae* and *V. eltor* (case) strains. From the present study it becomes evident that it also contains heat-labile antigens similar to those of *V. cholerae* and *V. eltor* (case) strains. The finding that the antigenic constituents of the proposed vaccine strain W 6 include the choleragenic toxin components is of importance in establishing that the vaccine strain is not devoid of residual virulence, an essential requirement for its use as a live vaccine. It also explains the genesis of the antitoxic immunity produced in the intestinal tract of adult rabbits following intra-intestinal administration of an apathogenic El Tor strain (Bhattacharya, Narayanaswami & Mukerjee, 1968).

SUMMARY

The antigens present in the preparation of cholera toxin consisting of the culture filtrate of 569 B strain of *V. cholerae* have been analysed by gel-diffusion, intra-gel absorption, and immuno-electrophoresis. The antiserum raised against the toxin was tested using the following antigens: whole cell lysates of *V. cholerae* 569 B and an El Tor (water) strain W 6 (proposed as a vaccine strain), suspensions of the same strains of vibrios with and without heat-denaturation, suspension of an El Tor (case) strain (GS-9) and a preparation of vibrio polysaccharide. The antitoxic serum gave four precipitin bands against the toxin preparation and nine bands against the whole cell lysate of *V. cholerae*. Two bands could be identified as being due to the heat-labile specific antigens present in the cholera toxin. These two bands were abolished on absorption of the antitoxic serum with a live suspension of W 6 but not on absorption with heat-denatured W 6. It has, therefore, been concluded that the proposed vaccine strain W 6 contains the specific antigens of cholera toxin. The significance of these results has been discussed in relation to the residual virulence of the proposed vaccine strain and antitoxic immunity produced in the intestinal tract of the adult rabbit following exposure to the vaccine strain.

We gratefully acknowledge the gift of purified vibrio polysaccharide from Dr C. V. N. Rao of the Indian Association for the Cultivation of Science, Jadavpur, Calcutta-32. The technical assistance of Mr B. Sanyamat and Mr I. Guhathakurta is also thankfully acknowledged.

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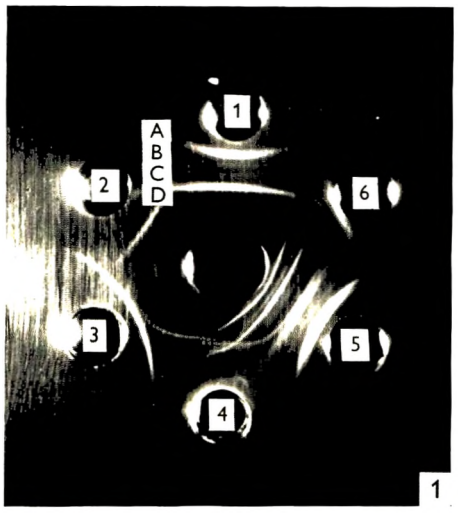
EXPLANATION OF PLATE

Figures 1–3.

Contents of wells	Well used in Fig. 1.	Fig. 2	Fig. 3
569B antitoxin	Centre	—	Centre
569B antitoxin, absorbed with heated W6 strain	—	Centre	—
569B toxin	1	1	1
Vibrio polysaccharide	2	—	—
Heated antigen of 569B	3	—	4
Live antigen of 569B	4	—	—
Whole cell lysate of 569B	5	2	2
Whole cell lysate of W6	—	3	6
Heated antigen of W6	—	4	5
Live antigen of W6	—	5	—
Live antigen of GS-9	—	6	3
Heated 569B toxin	6	—	—

Fig. 4. Antigen well contains 569B toxin. Trough contains antiserum against 569B toxin.


A
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C
D
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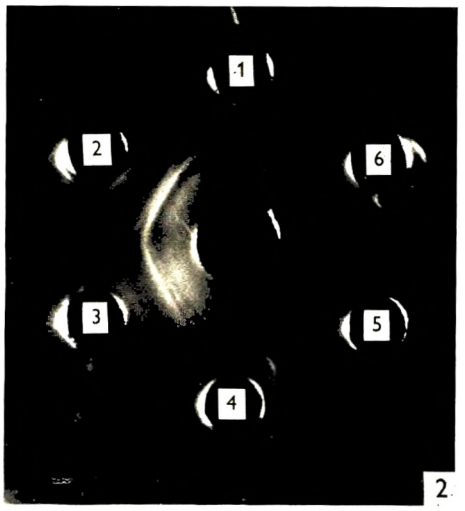
6
1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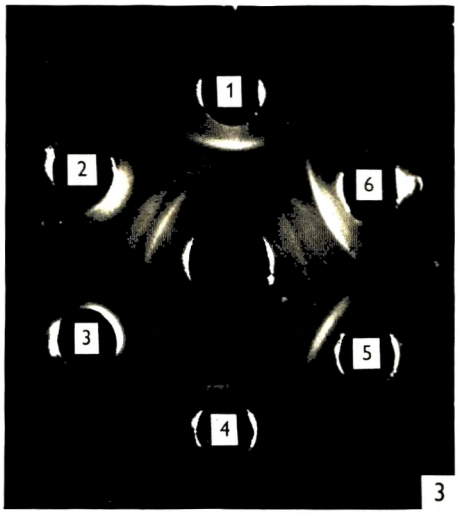
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4

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The isolation of parainfluenza 4 subtypes A and B in England and serological studies of their prevalence

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The term parainfluenza viruses was proposed for certain members of the myxovirus group which resembled the influenza viruses but which were not related to them antigenically (Andrewes, Bang, Chanock & Zhdanov, 1959). Included in this group were parainfluenza virus types 1, 2 and 3. Parainfluenza 2 (Croup associated or CA virus) was first described by Chanock in 1956, and parainfluenza 1 and 3 (Haemadsorption types 2 and 1 or HA-2 and HA-1) by Chanock and his colleagues in 1958.

In 1960 Johnson, Chanock, Cook & Huebner described a new haemadsorption virus which they called M-25. This strain was isolated from a college student and also from 30 infants in an orphanage nursery and was shown to be antigenically distinct from parainfluenza 1, 2 and 3 viruses. They proposed that M-25 should be the prototype strain for parainfluenza type 4.

In 1964 Canchola and his colleagues described a new strain which could not be identified with an antiserum prepared against the prototype parainfluenza type 4 (M-25) virus. They had isolated 25 strains but only two of these resembled M-25. The remaining 23 strains had a common complement fixing antigen but could be differentiated in neutralization tests. The two groups of strains were called parainfluenza 4 subtypes A and B respectively. For the first time also these workers were able to report an association of type 4 virus with respiratory disease in children.

In 1966 Zilisteanu and his colleagues reported the isolation of eight further strains of parainfluenza 4 subtype A. These were obtained from children in a nursery in Bucharest. Following an outbreak of measles, 12 of the children had mild respiratory signs, nasal obstruction, nasal catarrh, tracheitis and pharyngitis, but were afebrile and well, apart from these manifestations.

From these reports it appears that isolation of parainfluenza 4 virus is difficult because of the slow growth in cell culture and weak haemadsorption pattern and that infection with these strains produces a very mild respiratory illness mainly in young children.

Since 1962 in this laboratory all haemadsorbing viruses which could not be immediately identified have been stored at $-70°$ C. For the most part these strains came from other laboratories situated in different parts of the country.

This report is based on: (1) the identification of 18 strains of parainfluenza 4 virus, 15 being subtype A and 3 subtype B. (2) the clinical information available from these cases. (3) a serological survey to assess the prevalence of neutralizing antibody and these viruses in England.

MATERIALS AND METHODS

Prototype viruses

Prototype strains of parainfluenza 4 subtypes A and B were received from the National Institutes of Health, Bethesda, and were passaged in human embryo kidney and primary rhesus monkey kidney cultures. Customarily titres of 10^5 per 0·1 ml. were obtained.

Newly isolated strains

Only one of the parainfluenza 4 strains described here was isolated in this laboratory. It came from pooled nose and throat swab material of a child in a special respiratory survey. Seventeen strains were submitted for further study as unidentified haemadsorbing viruses in rhesus monkey kidney cell cultures. They were originally unidentifiable with the antisera then available, and were stored at −70° C. for later investigation.

All strains were passaged in primary rhesus monkey kidney cells maintained in Eagle's medium without serum. They were identified as parainfluenza 4 viruses by neutralization with antisera prepared against the prototype strains.

Preparation of specific immune sera

The sera were prepared in adult guinea-pigs which were bled before inoculation. An average of four guinea-pigs was used for each strain. Under light ether anaesthesia 0·2 ml. of virus suspension was inoculated intranasally. Ten days later a further 1 ml. of suspension was inoculated intraperitoneally and after a further 10 days the animals were bled out. Pre and post inoculation sera were tested simultaneously for antibody to the known human myxoviruses by cross neutralization tests. The titre of each serum to parainfluenza 4 A or B respectively was calculated.

Infectivity titrations

Serial tenfold dilutions of the virus suspensions were made in Earle's balanced salt solution (BSS) and 0·1 ml. of each dilution was inoculated into each of two tubes of primary rhesus monkey kidney cells. The cultures were rolled at 33° C. for 6 days and titres determined by the haemadsorption technique.

Identification of strains

The guinea-pig antisera were inactivated at 56° C. for 30 min. Doubling dilutions of each serum were then made in Earle's BSS and an equal volume of serum dilution was mixed with an equal volume of virus suspension calculated to contain 100 TCD_{50}/0·1 ml. Serum-virus mixtures were held at room temperature for 1 hr. and then 0·2 ml. of the mixture was inoculated into duplicate tubes of primary rhesus monkey kidney cell cultures. The tubes were rolled at 33° C. for 6 days and titres determined by the haemadsorption technique.

Neutralization tests on selected sera

Human sera from several sources were obtained as follows: (a) Sera from the London area submitted primarily for antistreptolysin (ASO) estimation. (b) Sera

from all age groups received from the Public Health Laboratory, Liverpool. (c) Sera from infants and young children submitted for rubella and cytomegalovirus studies.

Sera inactivated at 56° C. for 30 min. were screened for neutralizing antibody to parainfluenza 4 subtypes A and B at a dilution of 1/10.

In addition antibody titrations were done on paired sera from one of the patients from whom parainfluenza 4 subtype A was isolated.

RESULTS

Eighteen strains of parainfluenza virus type 4 have been identified; 15 resemble the prototype M-25 virus and are referred to as subtype A and three are strains of subtype B.

Table 1. *Strains of haemadsorbing virus received for identification*

Strain no.	From	Age	Admitted to hospital	Diagnosis
1/62	Manchester	12/12	Yes	Febrile cold
2/62	Manchester	3	Yes	NK
3/63	Bristol	18/12	Yes	Bronchitis
4/64	Prague	NK	NK	NK
5/64	Bristol	4	NK	Bronchitis
6/64	Oxford	21	No	URTI
7/64*	Stafford	7·6/12	No	Laryngitis
8/64	Bristol	3·4/12	Yes	Bronchopneumonia
9/65	Bristol	10/12	Yes	Bronchitis
10/65*	Manchester	4/12	Yes	Febrile convulsions Pleural effusion
11/67†	Manchester	12/12	NK	Rubella
12/67†	Manchester	5	No	Infectious erythema
13/67	Manchester	5	No	Contact of 12/67
14/67†	London	10/12	Yes	Bronchopneumonia Roseola infantum
15/67	London	2	No	Bronchitis
16/67	Leicester	10/12	NK	Febrile convulsions Tonsillitis
17/68*	London	5	No	Bronchitis
18/68	Oxford	3/52	Yes	Convulsions

* Parainfluenza 4 subtype *B* † Rash reported NK = not known

As shown in Table 1, these strains came from six different laboratories in England. The first strain to be identified in this country was from Czechoslovakia and was isolated in 1964. However, the first strain to be isolated in England was in February 1962 in Manchester.

Fifteen of the 17 English strains came from children under 6 years and nine were from children under 3 years. Clinical information was not available in all instances but Table 1 shows that eight out of 14 children were sufficiently ill to be admitted to hospital and nine out of 14 children had an infection of the lower respiratory tract, bronchitis or bronchopneumonia.

Strains 6, 7, 8 and 16 were from cases with pharyngitis and marked cervical

lymphadenopathy and strains 10, 16 and 18 from children with convulsions. One interesting clinical feature was that three strains, 11, 12 and 14 were isolated from children with rashes. They were diagnosed as having rubella, infectious erythema and roseola infantum respectively.

Paired sera were available from the patient from whom strain 6/64 was isolated. These sera showed a rise in antibody titre to parainfluenza 4 subtype A from < 10 to 80.

Four hundred sera were screened for neutralizing antibody to parainfluenza 4 viruses subtypes A and B and the results are shown in Tables 2 and 3.

Table 2. *Distribution of neutralizing antibody to parainfluenza* 4 *subtype A by age in* 399 *selected sera*

Age in years	No. tested	% with antibody
0–8/52	32	84
9/52–2	77	9
3–5	51	57
6–10	73	82
11–25	116	84
26–50	37	95
51–80	13	92

Table 3. *Distribution of neutralizing antibody to parainfluenza* 4 *subtype B by age in* 372 *selected sera*

Age in years	No. tested	% with antibody
0–8/52	29	65
9/52–2	75	7
3–5	50	52
6–10	74	65
11–25	94	64
26–50	37	76
51–80	13	70

From the distribution of antibody it would appear that, as with the other parainfluenza viruses, infection with both subtypes A and B is common in the pre-school child particularly in the age group 3–5 years, 57 % of the population having antibody to subtype A and 52 % to subtype B by the age of 5 years. After this age the distribution of antibody to type A and B varies somewhat with over 80 % of primary school children and over 90 % of young adults having antibody to type A, whereas with type B only 65–70 % of the adult population had antibody present.

DISCUSSION

Although strains of parainfluenza 4 virus have been found in England since 1962 they are infrequently isolated, a reason for this being the difficulty with which they are recovered from natural infections (Canchola *et al.* 1964). They require a

long incubation period for growth and on primary isolation little if any cytopathic change is seen. On passage these strains induce a distinctive cytopathic effect consisting of a granular rounding of cells, loss of cell outline, vacuolation of cells and in some cases a marked syncytial formation. Haemadsorption will often be demonstrable only at room temperature or 37° C, and because strains can best be recognized by haemadsorption some may be missed if the haemadsorption test is performed only at 4° C. The difficulties of virus recovery became apparent in a special respiratory survey by this laboratory when strains of parainfluenza 4 virus were specifically sought in a large children's home, where all age groups were represented and both severe and minor respiratory disease seen, but only one strain was recovered in 3 years from the 450 nose and throat specimens examined. It is therefore possible that other factors such as the presence of antibody or inhibitors may increase the difficulties of isolation of these viruses.

To see whether or not infection with parainfluenza 4 viruses really is infrequent a serological survey was done. This clearly established that antibody to parainfluenza 4 viruses was acquired early in life by a substantial proportion of the population.

Previous reports from America and Rumania suggested that infection with parainfluenza 4 viruses mainly causes a mild respiratory illness. In this country 9 out of 14 children from whom these viruses were isolated had involvement of the lower respiratory tract. Although the considerable immunity shown points to minor infections for the most part it seems probable that as with parainfluenza viruses types 1, 2 and 3, type 4 viruses may cause a range of illnesses from febrile colds to the more serious bronchitis and bronchopneumonia.

From the information presented parainfluenza 4 viruses could be important pathogens of respiratory disease in young children.

SUMMARY

The identification of 18 strains of haemadsorbing viruses as parainfluenza type 4 viruses subtypes A and B is reported, as is a serological survey to show the distribution of antibody to these viruses in various age groups. The results suggest that although isolation of the virus is infrequent, infection in early childhood is common and the viruses may be important respiratory pathogens.

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The influence of antigenic variation on influenza A2 epidemics

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(*Received* 14 *March* 1969)

The pattern of epidemics due to influenza A2 virus can be determined by several means: by following changes in sickness benefit claims, mortality rates and general practitioner consultation rates (Miller & Lee, 1969) and by accumulating laboratory evidence of the presence of influenza viruses in the population. Over the past decade information from these various sources has been found to correlate very closely and a clear picture can be drawn of the epidemics which have occurred every winter but three since 1957. The factors which allow such epidemics to develop are only partly understood but there are two which can be measured and which certainly play a part. One of these is the antigenic structure of the prevalent virus and the other is the antibody directed against it in the exposed population. Both these factors are unstable because of the antigenic variation which influenza A virus undergoes from time to time and because serum antibody is not necessarily maintained at high titre against the original infecting strain and, indeed, may become undetectable against a sufficiently changed new variant. Attempts have been made to assess the importance of these factors in explaining the repeated epidemics due to influenza A2 virus which have occurred in Britain since 1957.

MATERIALS AND METHODS

Virus strains

Strains of influenza virus isolated in public health and other laboratories by the inoculation either of fertile hens' eggs or of primary monkey kidney cell cultures were received at the Virus Reference Laboratory, Colindale, and after further passage were examined by haemagglutination-inhibition with specific ferret antisera. Prototype strains of influenza A2 variants were kindly supplied by Dr H. G. Pereira of the World Influenza Centre, Mill Hill.

Sera

Sera for the antibody survey were collected in October 1968 from the staff of a large London hospital for another study, and were kindly supplied to us by Dr G. ffrench. From this source 366 sera were obtained from persons aged between 16 and 67. For the younger age groups recourse was made to sera sent in for

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antistreptolysin O estimations at the end of 1968, and also to sera from children living in the south of England taken in 1968 for the investigation of pre- and post-natal rubella infection and kindly given by Dr E. Vandervelde.

Sera were stored at $-30°$ C until tested. Haemagglutination-inhibition tests were done as described by Pereira, Pereira & Law (1964).

RESULTS

Influenza viruses

Over the 11-year period since influenza A 2 virus first appeared, isolations of this virus have been made every winter with the exception of the winters 1959–60, 1961–2 (when only influenza B appeared) and 1966–7.

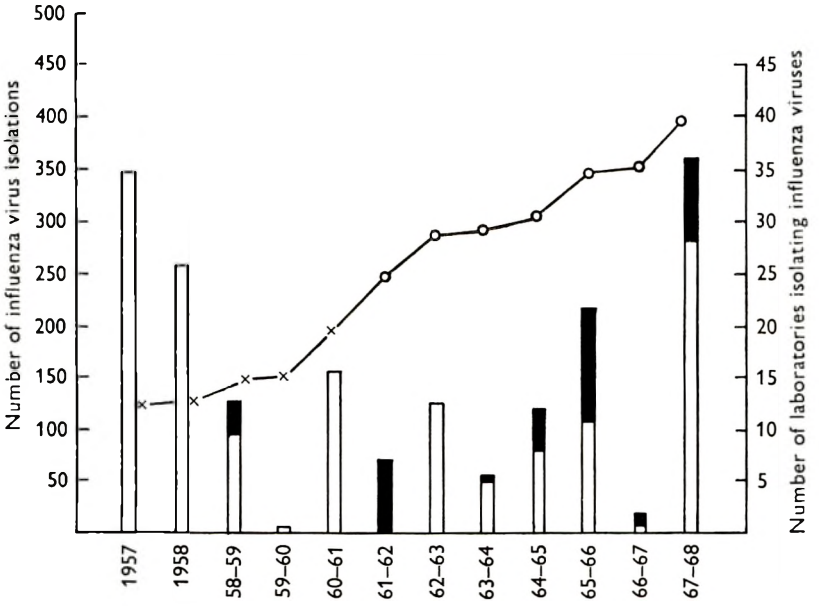

Fig. 1. Laboratories isolating influenza viruses and number of virus isolations, 1957–68. □ = Influenza A 2 isolations; ■ = influenza B isolations; × = laboratories using eggs; ○ = laboratories using monkey kidney.

Virus isolations usually extended from the end of December of one year to February or March of the next year. There have been two exceptions to this; one in 1957, when the epidemic began in September and continued with only a short break right through the winter to April 1958, and the other in the 1967–8 winter, when virus isolations were made unusually early, at the end of November of 1967, and continued through to March 1968.

The number of influenza A and B viruses which have been isolated and examined is shown in Fig. 1. Over the 11-year period this has varied considerably. The number does not reflect the extent of any epidemic, for not only has the number of laboratories undertaking virus diagnostic work increased but also techniques have been simplified, permitting a wider sampling of clinical cases.

The influenza A2 viruses isolated in each epidemic were tested against antisera to the original A2 strains and against representative antisera to each successive variant as these were detected. The first antigenic changes were noted in 1961 (Isaacs, Hart & Law, 1962) followed by reports of further differences in 1962–3 by Morris *et al.* (1963) and Weinberger, Buescher, McCown & Gauld (1963) and in 1964 by Pereira *et al.* (1964). The antigenic composition remained relatively stable from 1964 until 1967, when a strain was isolated in Tokyo differing recognizably from those which had circulated during the previous 3 years. This variant, A2/Tokyo/3/67, was isolated in outbreaks of influenza in many countries in the world that year. In Britain, in the epidemic of the winter of 1967–8, it accounted for about one-fifth of the influenza viruses isolated, the remainder being antigenically similar to the earlier strains. Another variant A2/Hong Kong/1/68, even further altered, was isolated in 1968 first in Hong Kong and Singapore and rapidly spread to many parts of the world.

Table 1. *Comparison of influenza A2 viruses by haemagglutination-inhibition*

	Ferret sera					
Virus	A2 Singapore 1/57	A2 England 43/63	A2 England 12/64	A2 England 68/68	A2 Tokyo 3/67	A2 Hong Kong 1/68
A2/Singapore/1/57	**5120**	2560	120	1280	< 10	160
A2/England/43/63	1280	**5120**	240	2560	320	120
A2/England/12/64	160	1280	**320**	5120	120	20
A2/England/68/68	640	2560	640	**5120**	60	120
A2/Tokyo/3/67	160	320	40	240	**480**	< 10
A2/Hong Kong/1/68	640	160	20	640	< 10	**2560**

The antigenic differences between all these variants are shown in Table 1. The difference between the 1957 and the 1963 strains was demonstrable but of small degree. With the 1964 prototype strain it was found that while its antiserum inhibited the earlier A2 viruses moderately well, the strain itself was only poorly inhibited by antiserum to the 1957 strain.

From 1964 small alterations occurred until 1968, when the Tokyo variant appeared in Britain, and this strain, while clearly an A2 virus, showed a more marked shift. This antigenic difference is magnified in Table 1 because of the low homologous titre. Both this strain and the later Hong Kong/68 strain showed a further shift away from the earlier A2 viruses, although they still had components common to them. It is striking that the two most recent variants show no cross-reactivity, one with the other.

An interesting finding related to the growth properties of the successive variants of influenza A2 virus has been the gradual increase in difficulty of isolating and propagating these viruses in fertile hens' eggs. In 1957 the method commonly used for the isolation of influenza virus was the inoculation of such eggs. Viruses were often detected on the first passage and could be grown to high titre on further passage. Primary monkey kidney cell culture had largely replaced egg isolation by 1961 but strains thus isolated could be passaged with ease in eggs. In later years it

became more difficult to isolate strains in eggs and also to passage monkey kidney isolates in eggs. By contrast the Hong Kong/68 variant has been easy to isolate in eggs, yielding high titres of haemagglutinin and behaving more like the original A2 strains than those which have been circulating in the intervening years.

Influenza antibodies

A considerable proportion of the population became infected during the widespread epidemics in 1957, 1958 and 1959 and small surveys after these three epidemics indicated that over half the serum samples examined contained antibody to the Singapore/1/57 strain of influenza A2 virus. The quiet winter of 1959–60 was followed, however, by an extensive outbreak in 1960–1, an outbreak which further increased the proportion of the population with antibody. The antigenic changes detected in the virus at this time were only slight. In the succeeding years the epidemics which occurred were less extensive despite the further antigenic shift found in the A2 viruses isolated in 1964.

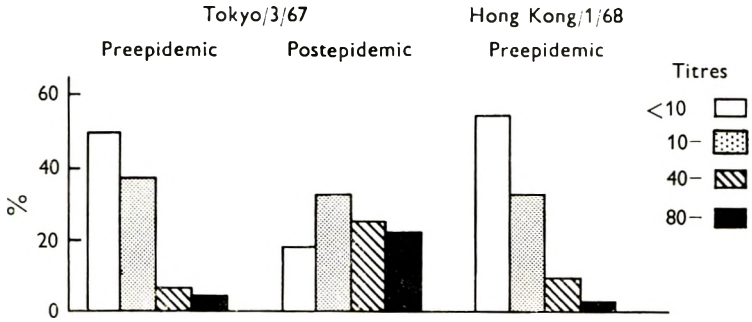

Fig. 2. H.I. antibody to influenza A2 variants, all ages.

A more comprehensive survey of antibody undertaken in 1966 (Pereira, Chakraverty, Pollock & Pope, 1967) again indicated the presence of antibody in a substantial proportion of the population and accordingly epidemics due to the strain then circulating seemed unlikely. The winter of 1966–7 was free from influenza but in the next winter of 1967–8 there was an epidemic of some size. In this epidemic viruses of two antigenic varieties were isolated; one of these was similar to the strains which had circulated during the previous year, the other, which occurred less frequently, was similar to the newer variant A2/Tokyo/3/67. Retrospective studies made on sera taken before the appearance of this variant indicated a possible explanation for this epidemic. The results of these tests are shown in Fig. 2. It can be seen that the proportion of people with high antibody titres to the Tokyo/67 variant was small before the epidemic, whereas 2 years later after the epidemic, the proportion with high titres had greatly increased. The pre-epidemic pattern of antibody to the Tokyo/67 variant was clearly similar to the pattern of antibody to the Hong Kong/68 variant in sera taken before this strain appeared in Britain.

The A2 variant isolated in Hong Kong in 1968 displays an antigenic difference

from the previous A2 strains even more marked than did the Tokyo variant of 1967. The occurrence of outbreaks of influenza in many parts of Asia following its first appearance suggested that antibody to this strain must be low or absent in these populations and sera recently collected in England from people of different ages were therefore tested to estimate the proportion with antibody in Britain. Previous studies (Pereira *et al.* 1967) had shown that very small differences were demonstrable in the antibody pattern in people from widely separated areas in this

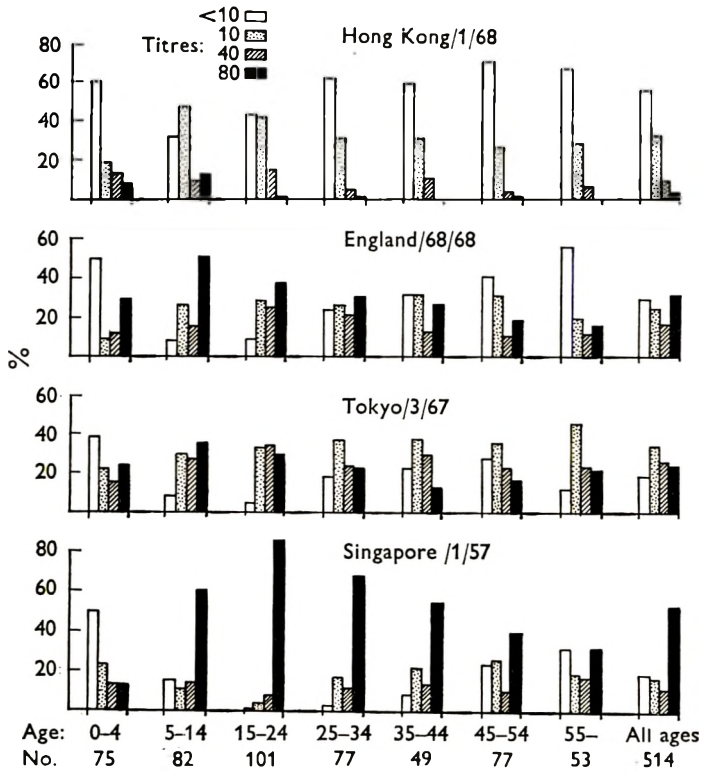

Fig. 3. H.I. antibody to influenza A2 variants among different age groups.

country, so, although most of the latest sera tested were from people in the south-east of England, it is likely that the picture would be similar for the rest of the country. The age distribution and titres of antibody to the Hong Kong/68 variant and to three other A2 strains are shown in Fig. 3. It can be seen that antibody to the original A2/57 virus was present not only in a high proportion of all groups except the under-5-year-olds, but was also present at titres of 1/80 or higher. Antibody to the two strains prevalent in the 1967–8 epidemic represented by A2/England/68/68 and A2/Tokyo/3/67, although present in a high proportion of sera, was found at lower titres compared with the A2/Singapore/1/57 strain. The contrast between these results and those obtained with the A2/Hong Kong/1/68 strain is striking. With this virus the proportion of persons with no detectable antibody is 60% or more in all adults and in those under 5 years of age, and the number with titres

above 1/80 is very small. It is noticeable that in the younger age groups, school-age children and young adults, more than half had antibody at titres of 1/10 or higher. The more frequent detection of antibody to the two recent variants in the younger age groups suggests that these young people (since many of them were not alive at the time of the biggest epidemics due to the original A 2 virus) have been infected more recently with variants antigenically closer to the new strains.

DISCUSSION

Predictions of the amount of illness due to influenza virus during any winter are greatly affected by the antigenic composition of the viruses which will circulate in the population, and the amount of the appropriate antibody available in the population. With a new sub-type the prediction is simple, since the population is not immune and spread of the virus is almost certain. In successive years, as antibody becomes more generally acquired, epidemics, although they occur, become smaller in size and duration and this situation may persist over several winters. The appearance of variants changes the pattern and allows the virus to attack those who have already been infected by the same sub-type but whose antibody has perhaps fallen to unprotective titres. Exactly what these titres have to be is one of the points on which firm evidence is scanty. Meiklejohn, Kempe, Thalman & Lennette (1952) were able to make some estimate in a study where antibody titres in military personnel were related to attack rates in a subsequent epidemic due to Influenza A 1 virus. The attack rate when the haemagglutination-inhibition antibody titre was $< 1/8$ was estimated at 18·3 %, at 1/8 the rate fell to 7·6 % and at 1/16 to only 1·5 %. In the epidemic of 1967–8 the lack of antibody to the Tokyo/67 variant may have allowed this virus to infect many who had antibody to the early A 2 strains and this could well have contributed to the somewhat unexpected size of the epidemic.

The position with the Hong Kong/68 variant is similar in that antibody is absent in a large proportion of the population. However, if as has been suggested by Meiklejohn, low antibody titres do afford protection, the presence of antibody found among the younger age groups in this country could well have a modifying effect on an epidemic, particularly since these groups are suspected of playing a significant part in the dissemination of infection.

SUMMARY

The antigenic variation of influenza A 2 virus and the antibody present in the population to different variants are described and related to the occurrence of epidemics of influenza in England since 1957.

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Influenza in Britain 1967–68

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INTRODUCTION

During the 35 years since the discovery of the influenza virus, its laboratory properties, clinical effects and epidemiology have been widely studied and much effort has been devoted to the development of vaccines. Yet influenza remains virtually uncontrolled, and, during epidemics, seriously disrupts the activities of affected communities and causes many deaths; it is still impossible to predict with accuracy when and where epidemics will occur and no satisfactory treatment is known. Much of the difficulty lies in the frequent changes in antigenic composition that the virus undergoes, and in the fact that immunity to one variant may confer only partial immunity to another. Efficient schemes of national and international influenza surveillance are therefore important in providing early warning of the appearance and spread of new antigenic variants and to assess the extent of immunity to them; this information, if obtained quickly enough, may permit the production of suitable vaccines and their use in advance of an epidemic to protect the most vulnerable population groups.

Consideration of the past behaviour of influenza may be helpful in interpreting surveillance reports and in judging the likely behaviour of influenza in particular circumstances. This paper sets out, therefore, to provide a brief review of experience of influenza in Britain since the last major antigenic variant of influenza virus A, the Asian (A2) virus, was introduced in 1957, and to examine in more detail the epidemiology of the epidemic of the winter 1967–8, which was the largest for 7 years.

SOURCES OF EPIDEMIOLOGICAL INFORMATION

Several independent sources of information that reflect the prevalence of influenza have been selected for analysis, including mortality statistics, sickness benefit claims, general practitioner records and laboratory reports. The analyses include figures for England, Wales and Scotland combined, unless otherwise stated.

Deaths

The number of deaths attributed to influenza (International Classification of Diseases (ICD) Nos. 480–3) for the years 1957–68 were extracted from the Registrar Generals' weekly returns; these returns record deaths up to and including Friday of each week. Deaths due to pneumonia (ICD Nos. 490–3) and bronchitis (ICD Nos. 500–2) during the winter 1967–8 were also analysed; pneumonia deaths do

not include those secondary to accidents and other infections; bronchitis deaths include both acute and chronic bronchitis.

The age of distribution of influenza deaths in the 1967–8 epidemic was obtained from the (then) Ministry of Health's weekly influenza statement; these figures were based on provisional notifications to the Registrar General (England and Wales only).

Sickness benefit claims

Claims for sickness benefit under the National Insurance Act are submitted by insured persons to the Department of Health and Social Security (formerly the Ministry of Pensions and National Insurance and, later, the Ministry of Social Security), and the numbers of new claims received in each of the standard statistical regions of England, Wales and Scotland up to and including Tuesday of each week, are published in the Registrar Generals' weekly returns. The certified causes of absence are not published in these returns, and analyses could be made only of the total numbers of claims.

Royal College of General Practitioners' morbidity returns

The Records and Statistics Unit of the Royal College of General Practitioners receives returns from general practitioners in various localities, which record the number of new sickness episodes in their practices according to age, sex and clinical diagnosis of patients, during the 7 days up to and including Wednesday of each week. During the winter of 1967–8 these returns were submitted from 40 general practices in England and Scotland (23 urban, 10 rural and 7 mixed) with a total population of approximately 150,000. Weekly rates for cases of influenza and other acute respiratory illnesses were extracted from these returns. Except where otherwise stated, the term 'influenza' when applied to case rates reported by general practitioners includes illnesses diagnosed on clinical grounds as influenza or influenza-like or febrile common cold. The diagnosis is not normally supported by laboratory evidence of influenza virus infection.

Laboratory reports

Influenza virus isolations and cases with serological evidence of recent influenza virus infection (four fold or greater rise in antibody titre) are reported each week by virus diagnostic laboratories in Britain to the Public Health Laboratory Service. These reports state the date on which the specimen was received by the laboratory, the virus serotype identified, the age and sex of the patient, and the main clinical features of the illness. The selection of patients for virological investigation depends entirely on clinical considerations and local epidemiological interest, and the submission of specimens may be stimulated by knowledge that influenza is likely to be present in the community. Thus the nature of the population sampled for laboratory studies cannot be defined, but the viruses identified probably broadly represent the types present in the population.

PREVALENCE OF INFLUENZA IN BRITAIN 1957–68

Figure 1 traces the average weekly number of deaths attributed to influenza for each month and the average weekly number of new claims for sickness benefit in England and Wales for each month from 1957 to 1968. The types of influenza virus shown by current laboratory reports to be prevalent during each year when influenza was epidemic are also shown in this figure.

Each winter there was a seasonal rise in the number of sickness claims, but in the winters when there was a large number of influenza deaths and laboratories were reporting significant numbers of influenza virus infections, the number of claims greatly exceeded that in epidemic-free years. The increase in claims usually slightly preceded or coincided with a correspondingly sharp increase in influenza deaths. It appears, therefore, that the excess number of claims in epidemic years is probably largely due to influenza and may be taken as an approximate index of influenza morbidity, at least in the insured population.

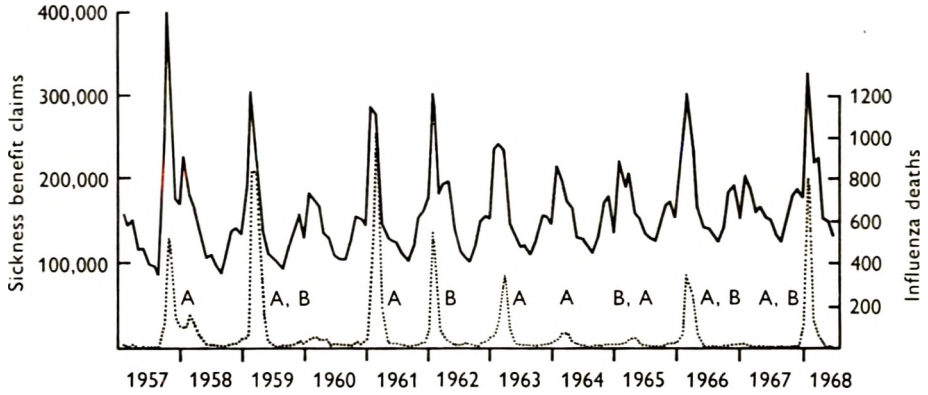

Fig. 1. Influenza in England and Wales, 1957–68. Average weekly no. of new sickness benefit claims and average weekly no. of influenza deaths (in four-weekly periods). ———, New sickness benefit claims; ·····, influenza deaths.

Virus A2 (Asian) influenza was first epidemic in Britain during the autumn of 1957 and again in early 1958. Further major epidemics of influenza A occurred in 1959, 1961, 1963, 1966 and 1968, with smaller epidemics in 1964 and 1965. Influenza virus B was isolated during the epidemics of 1959, 1962, 1965, 1966 and 1968. In gauging the size of recent epidemics from Fig. 1 it should be noted that during the period as a whole the number of sickness benefit claims tended to increase irrespective of seasonal or epidemic changes. Thus the summer trough rose by approximately 40,000 claims between 1957 and 1967. No regular pattern of recurrence of influenza A epidemics is evident, but between 1961 and 1966 the size of outbreaks, as indicated by numbers of sickness claims and by numbers of deaths, appeared to be diminishing. This trend was interrupted by a moderately large outbreak in the winter of 1965–6 and by the even larger epidemic in 1967–8. In the intervening years influenza A, when present, was largely confined to local community or institutional outbreaks and sporadic cases. Influenza B showed a more regular pattern of recurrence, appearing every 3 years, with the exception of

1965 and 1966 when it was present in successive years. In each year when virus B occurred concurrently with A, except in 1965, fewer B than A infections were identified by laboratories. Virus B is sometimes said to be less severe than A, but in 1962, a year when it was the sole cause of the epidemic, it is notable that there were as many deaths as in some influenza A outbreaks of similar size.

THE 1967–8 EPIDEMIC

World-wide influenza

Reports in the World Health Organization's weekly epidemiological record indicated that countries in the northern hemisphere, with the exception of certain Eastern European States, experienced little influenza during the winter of 1966–7. In July and August 1967 outbreaks of influenza A2 were reported from South Africa and New Zealand. Strains isolated proved to be antigenically similar to those that had been circulating in Britain in the past few years. As antibody to this variant and to influenza B appeared to be well distributed throughout the population (Pereira, Chakraverty, Pollock & Pope, 1967) widespread outbreaks were not expected.

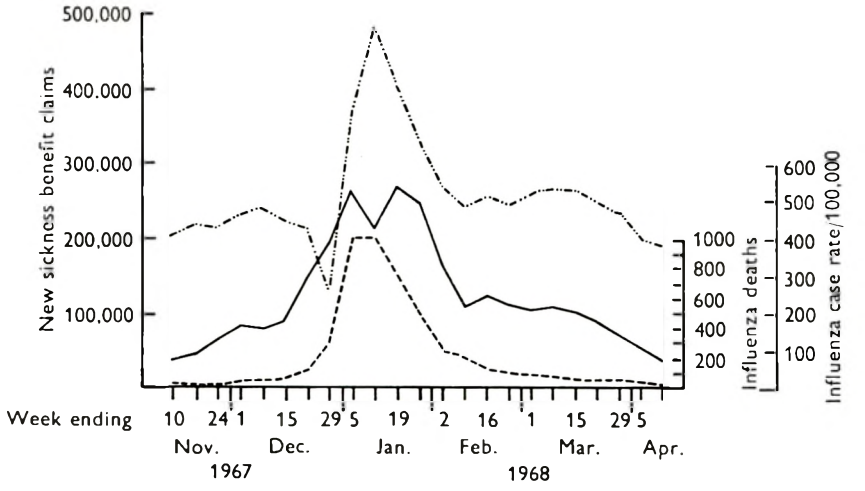

Fig. 2. Weekly nos. of sickness benefit claims and influenza deaths with influenza case rates reported by general practitioners. England, Wales and Scotland, November 1967 to April 1968. –·–·–, new sickness benefit claims; ——, influenza case rates; – – – –, influenza deaths.

The first of the winter's outbreaks of influenza in the northern hemisphere were reported from Michigan, U.S.A. in school children and university students during October and November 1967; thereafter influenza spread to affect the whole country. Influenza began in many countries of Europe, including Britain, in December, and extensive epidemics, nearly all due to virus A2, ensued.

The epidemic in Britain

It has been noted that the epidemic during the winter 1967–8 was the largest for 7 years (Fig. 1). A detailed picture of the progress of this epidemic is shown in

Fig. 2, which charts weekly numbers of influenza deaths and sickness benefit claims, and rates for cases of influenza reported by general practitioners. The recording weeks covered by returns from these three sources unfortunately do not exactly coincide; in this graph the difference has been ignored and each has been plotted against the dates for the weeks ending on Friday, which are those used for the Registrar General's returns of deaths; in fact, the general practitioners' returns cover the week ending 2 days earlier and the sickness benefit returns the week ending 3 days earlier.

The graphs show that the main epidemic began towards the end of December and was over by early February; but all three indices began to rise about 1 month earlier and did not return to the mid-November levels before the end of March. Increased morbidity and mortality rates from respiratory disease are to be expected

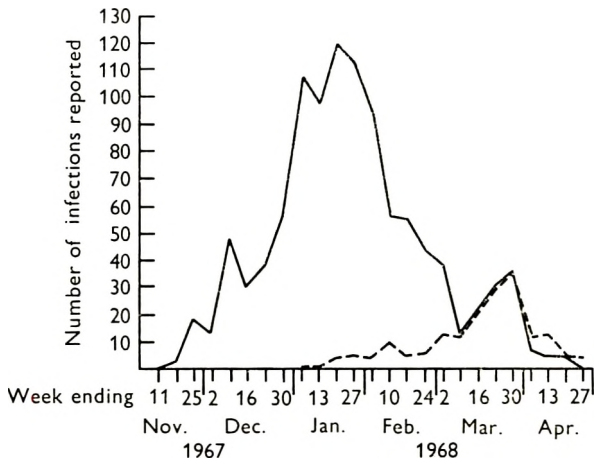

Fig. 3. Weekly no. of influenza virus infections identified by laboratories, November 1967 to April 1968. ——, influenza A; – – – –, influenza B.

at this time of year and some illnesses and deaths diagnosed as due to influenza may be due to other causes. But Fig. 3 shows that laboratory reports of influenza, based on the week in which specimens were received, also began to accumulate in late November and continued until the end of March, with a few as late as April. Although trends based on laboratory reports must be interpreted with caution owing to the uncertain method of selecting cases for examination, the number of influenza infections reported followed a general pattern similar to that in Fig. 2. The main epidemic from December to February was evidently caused by virus A, but in March an increased number of virus B infections was reported with a small recrudescence of virus A infections.

The sharp fall in sickness benefit claims in the last week of December coincided with the Christmas holidays, which fell on 2 week-days, and may be accounted for in this way. Thereafter claims rose steeply to a peak in the second week of January, a week later than the peak in case rates in general practice and in deaths; this may be due to the late submission of claims after the holidays. After the second week of January the number of sickness claims and deaths declined rapidly, but influenza

case rates in general practice and the number of laboratory reports of influenza, after a small fall in the second week, showed a second peak in the third week before declining.

The geographical spread of the epidemic

A few, mainly sporadic, cases of influenza A and B infection were reported by laboratories in various areas in October and early November 1967. The first reports of community outbreaks, supported by influenza A virus isolations, came from the Chester and Liverpool areas, in the north-west of England during the last week of November. Outbreaks due to this virus were also reported in two residential institutions in Yorkshire, but there was no evidence of influenza in the general population of Yorkshire at the time. In the south of England, influenza was first reported in early December, when virus A was isolated among children and staff in a residential and day nursery in North London. Laboratory reports of influenza A simultaneously in many parts of the country quickly followed.

The extent of the epidemic in different parts of the country may be gauged by analysing the weekly numbers of sickness benefit claims in each of the standard statistical regions. This analysis showed that there was an increase in claims in the north-west region at the end of November and beginning of December, confined mainly to the Wirral and south-west Lancashire, coinciding with laboratory reports of outbreaks in these areas, but no substantial increase occurred in any other area before Christmas. After Christmas the number of claims everywhere increased rapidly, reaching a peak in the first week of January, except in Scotland, where both the increase and the peak were recorded a week later (presumably because of the New Year holiday in Scotland). Claims then declined steeply in all regions, though the decline was noticeably slower in the south-west than elsewhere. In all regions claims returned to their early November numbers by the end of March or the beginning of April.

Age groups affected

Figure 4 shows weekly influenza case rates reported by general practitioners by age from the end of November to the end of February. In adults, rates began to increase from early December and reached a peak in the first week of January. Rates in persons over 65 years of age did not rise as high as those in younger adults, but continued at an increased level for longer. In children aged 5–14 years, rates rose in the first 3 weeks of December and then declined, when schools closed for the Christmas holidays. However, in the week ending 17 January, after the reopening of the schools, there was a further sharp rise in the rate to a higher peak than that reached in this age group in December. The rates in preschool children, under the age of five, showed the biggest rise of any age group in late December and reached a peak in early January; this was followed by a second peak, coming after the second rise in older children, in late January. During this period laboratories reported increased numbers of isolations of respiratory syncytial virus (R.S.V.) as well as of influenza mainly from children under the age of 5 years. The increased prevalence of R.S.V. began in November, earlier than influenza, and continued

until the end of January; R.S.V. infections may therefore account for some of the cases of clinical influenza diagnosed at this time in young children. No other respiratory virus infections were reported in large numbers.

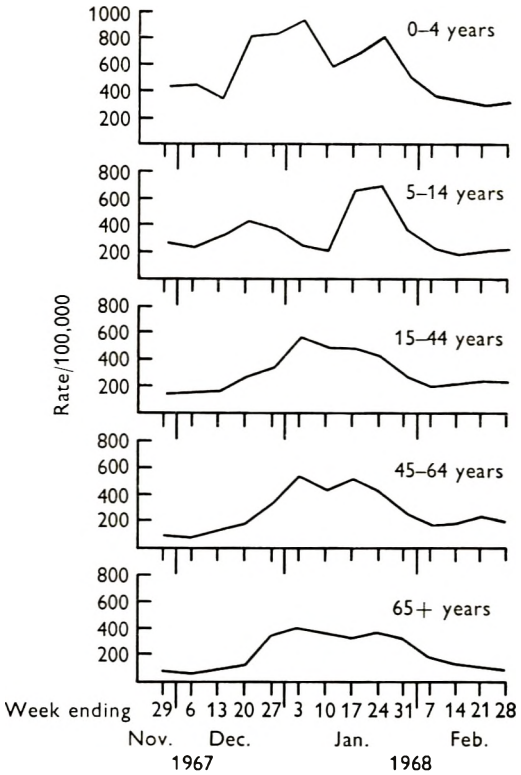

Fig. 4. Weekly influenza case rates by age reported by general practitioners.

Table 1. *Age-specific consultation rates for influenza reported by general practitioners*

(Weeks ending 29 November 1967 to 28 February 1968 inclusive.)

Age (yrs.)	Rates/thousand
0–4	76·0
5–14	46·3
15–44	40·6
45–64	36·6
65 or more	27·9

Table 1 shows the age-specific consultation rates in general practice. The rates in young children were considerably higher than in older children and in adults. However, Fig. 4 shows that the rates in children were higher than in adults before the epidemic, which suggests that the figures for children include a higher proportion of illnesses not due to influenza virus infection. Considering only illnesses diagnosed as influenza there was little difference in the consultation rates between age groups, but this may underestimate the infection rate. The age distribution of cases

in which laboratory evidence of infection was found (see Fig. 3) is shown in Table 2. The proportion of cases sampled may have varied between age groups and the age distribution of virus isolations does not necessarily reflect that of all cases. However, it may be noted that in influenza A infections all age groups were equally represented, except those under the age of four, who contributed approximately one-sixth of the total. The distribution of influenza virus B infections was different: 30 % of the total was in children aged 5–14 years and there was a relative deficiency of cases in older adults.

Table 2. *Age distribution of laboratory-diagnosed cases of influenza A and B infection*

	No. of cases		Distribution (%)	
Age (yrs.)	A	B	A	B
under 2	90	10	8·6	5·6
2–4	74	19	7·1	10·6
5–14	118	54	11·3	30·2
'Child'	1	—	0·1	—
All children	283	83	27·2	46·4
15–44	336	66	32·3	36·9
45–64	179	13	17·2	7·3
65 and over	157	5	15·1	2·8
'Adult'	9	—	0·9	—
All adults	681	84	65·4	46·9
Not stated	77	12	7·4	6·7
All ages	1041	179	100	100

Clinical features of illness

Comparison of the incidence of influenza with that of other respiratory illnesses reported by general practitioners (Fig. 5) showed that the rate of increase in cases of pneumonia in December and early January was approximately parallel to that in cases of influenza, which suggests that much of the increase in pneumonia was primarily due to influenzal infection. The incidence of bronchitis, tracheitis and laryngitis also increased during the epidemic but was not as closely associated with that of influenza.

The main clinical features of illnesses in which influenza virus was identified are shown in Table 3. Cases selected for virus examination may not be representative of all respiratory illness caused by influenza viruses; in particular, they are likely to include a high proportion of patients suspected of having influenza infection on clinical grounds and those who are most seriously ill. But it is interesting to compare the clinical features of influenza A and influenza B infections, which are presumably selected on a similar basis. Influenza A infections were more often diagnosed clinically as influenza and more often associated with lower respiratory illnesses than influenza B, but were rather less often recorded as upper respiratory illness. The proportion of cases in which lower respiratory illness was the main feature was

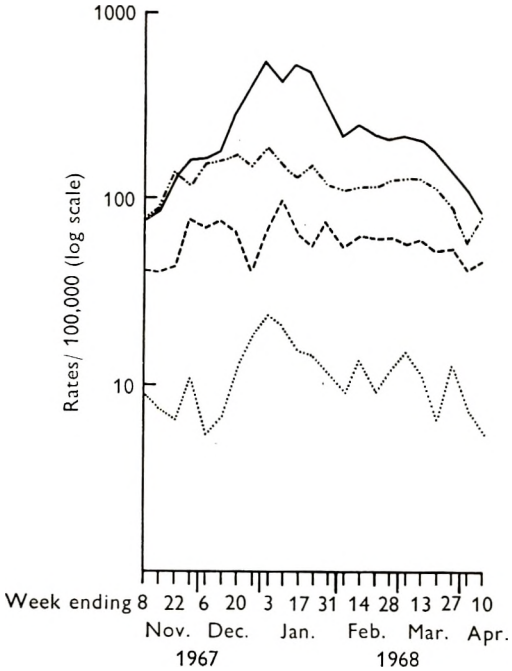

Fig. 5. Weekly case rates by diagnosis reported by general practitioners. ——, influenza; –·–·–, acute bronchitis; – – –, laryngitis and tracheitis; ·····, pneumonia and pneumonitis.

Table 3. *Main clinical features in laboratory-diagnosed cases of influenza A and B infection*

	No. of cases		(%)	
Main clinical features	A	B	A	B
Influenza-like illness	574	70	55·1	39·1
Upper respiratory illness (cold, coryza, pharyngitis, sinusitis, tonsillitis)	121	34	11·6	19·0
Lower respiratory illness (laryngitis, tracheitis, bronchitis, pneumonia)	264	38	25·4	21·2
General non-specific symptoms (pyrexia, headache, myalgia)	27	11	2·6	6·1
Central nervous system				
Convulsions	1	—		
Encephalitis	2	1		
Meningitis	8 (13)	1 (4)	1·2	2·2
Meningism	1	1		
Neuritis	1	1		
Gastro-intestinal symptoms (Vomiting, diarrhoea, abdominal pain)	6	1	0·6	0·6
Others*	36	21	3·5	11·7
Total	1041	179	100	100

* No symptoms, no information, unspecified respiratory or CNS illness.

highest in young children, particularly those under two, and in adults over 65 years (Table 4); at these ages such cases amounted to more than half the total of both influenza A and influenza B infections.

Table 4. *Frequency of lower respiratory complications by age group in laboratory-diagnosed cases of influenza*

Age (yrs.)	Total diagnosed	No. with lower respiratory illness	(%)
under 2	100	53	53·0
2–4	93	37	39·8
5–14	172	21	12·2
'Child'	1	1	100·0
All children	366	112	30·6
15–44	402	34	8·5
45–64	192	64	33·3
65 and over	162	87	53·7
'Adult'	9	1	11·1
All adults	765	186	24·3
Not stated	89	4	4·5
All ages	1220	302	24·8

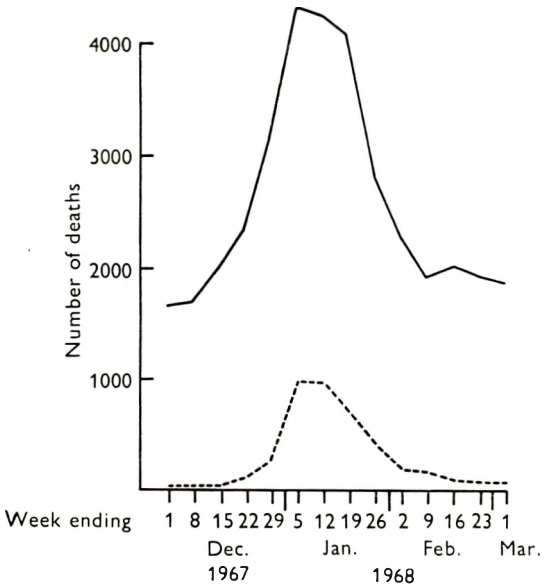

Fig. 6. Weekly no. of deaths from influenza, bronchitis and pneumonia, for the period December 1967 to March 1968. – – – –, influenza; ——, bronchitis and pneumonia.

Mortality

During the 4 months from the beginning of December to the end of March there were 4503 deaths certified as due to influenza in England and Wales and 145 in Scotland. Figure 6 compares the weekly numbers of deaths attributed to influenza with those attributed to bronchitis or pneumonia. The increase both in

bronchitis and in pneumonia deaths followed a similar pattern to that of influenza deaths. Thus, it seems likely that influenza virus infection was the primary cause of many of the deaths attributed to bronchitis and pneumonia in addition to those diagnosed as influenza. Table 5 records the age distribution of 4134 influenza deaths in England and Wales from the end of December 1967 to the end of February 1968. Most deaths occurred in older persons, mainly in those over the age of 65; there were only 27 deaths attributed to influenza in children.

Total incidence of influenza

It is difficult from the figures quoted to estimate the actual incidence of influenza virus infection in different population groups or the population as a whole. Only a large prospective study, supported by laboratory investigations could do so. However, the excess mortality and morbidity attributable to influenza during the winter 1967–8, when an epidemic took place, may be estimated by comparison with similar figures in an epidemic-free winter, 1966–7.

The number of deaths attributed to influenza from December 1967 to March 1968 (inclusive) exceeded those for same period in 1966–7 by 4444; the excess for pneumonia was 10,595, for bronchitis 6579 and for all causes 46,408. Similarly the difference between the number of sickness benefit claims in the two winters provides an approximate figure of the number of influenza cases in the working population. The excess number of claims in 1967–8 over those in the winter of 1966–7 in England and Wales and Scotland for the 4 months December to March was just over 1·1 million, a figure similar to that noted in previous epidemics (Roden, 1963). This, however, excludes cases in children, and in non-insured and retired adults. From a similar calculation based on the attack rates reported by general practitioners it is estimated that in the total population of England, Wales and Scotland there was an excess of 1·9 million cases diagnosed as influenza in the period December to March 1967–8 compared with the corresponding period 1966–7.

Table 5. *Age distribution of influenza deaths*

(Weeks ending 29 December 1967 to 1 March 1968, inclusive.)

Age (yrs.)	No. of deaths	Distribution (%)
0–4	19	0·5
5–14	8	0·2
15–44	63	1·5
45–54	76	1·8
55–64	295	7·1
65–74	772	18·7
75 and over	2901	70·2
All ages	4134	100

DISCUSSION

Epidemics of influenza cause large and sudden increases in morbidity and mortality in all sections of the population in a way that no other infection does. Because the impact of influenza is so dramatic any of a number of different

indices may be used for surveillance purposes. The way in which several such indices reflect the presence of epidemic influenza is illustrated by the analyses presented in this paper. Each gives a different kind of information and, though none by itself is entirely satisfactory, taken together they do provide a fairly complete picture of the progress, extent and seriousness of an epidemic. Also, provided adequate base-line information is available about the level of reporting in non-epidemic periods and rapid central collection and analysis of information is ensured, each is sufficiently sensitive to detect the early stages of an epidemic. Unfortunately, owing to the rapid evolution of epidemics locally the warning usually comes too late to be useful in the area concerned, but it may give at least some notice to medical services elsewhere that an epidemic could be imminent. Thus, the outbreaks reported in the north-west of England in early December 1967 presaged the nationwide epidemic later in the month. It is not clear what factors determine the spread of an epidemic. According to the sickness benefit figures, after the outbreaks in the north-west there was an interval with little evidence of widespread sickness in the population, followed by explosive epidemics simultaneously in all parts of the country. If this is a true description of the epidemic it suggests that the virus was widely disseminated during the lag period but only began to cause illness when some undefined environmental conditions were favourable.

Mortality attributed to influenza probably includes some cases not infected with influenza virus, and, conversely, some deaths certified as due to bronchitis, pneumonia and probably some other causes such as heart failure, are in fact primarily due to influenza. This suggestion is borne out by the observation during epidemics in the United States of excess deaths, not only in persons with acute respiratory disease, but also in those with cardiovascular or renal disease, some bronchopulmonary diseases and diabetes mellitus (Eickhoff, Sherman & Serfling, 1961). This would account for the fact that mortality from all causes during the epidemics exceeds the seasonal expected rates in non-epidemic periods and that the excess is greater than can be explained by influenza deaths alone (McDonald, 1963, 1967). Moreover, at these times the increase in deaths from all causes tends to run parallel with that of influenza deaths (Roden, 1963) again suggesting that the excess is directly due to influenza. The findings reported in this paper give further evidence that influenza causes many deaths other than those so certified. Fortunately lack of diagnostic precision in recorded causes of death does not seriously detract from the value of mortality rates in influenza surveillance. The same is evidently true of morbidity returns from general practitioners: two groups of diagnoses, those recorded as influenza and those recorded as influenza-like illnesses or febrile common cold each showed a similar pattern and because of this were combined in the analysis. Among other diagnoses changes in pneumonia rates followed those of influenza most closely, but other diagnoses also increased. The increase in other diagnoses was most noticeable in the early stages of the epidemic, possibly because as the epidemic progressed awareness that influenza was prevalent led to this diagnosis being used with increasing frequency. Thus, the consultation rate for *all* acute respiratory disease may be as useful an index of the early stages of an epidemic as that for influenza alone and possibly more so.

As expected, the great majority of influenza deaths were in persons over the age of 65, and lower respiratory complications were commoner in young children and older adults. Age-specific attack rates without laboratory confirmation are difficult to interpret, particularly since there was evidence that the diagnosis was less precise in children, especially those under 5 years of age, than in adults. But there was no clear evidence of greater susceptibility to influenza A in particular age groups. It may be significant, however, that influenza B was detected most often in children of school age, possibly owing to the fact that the last major epidemic of influenza B was in 1962. It is often stated that schools form the main source of spread of infection. The epidemic of 1967–8 spanned the school Christmas vacation and it is clear that the epidemic in children of school age was interrupted for the duration of the break. There is no evidence, however, that this influenced to a noticeable extent the course of the epidemic in adults or in preschool children, except that there was a second wave of cases in the under five-year-olds after the older children returned to school. The smaller, but more prolonged increase in rates in the elderly may simply reflect their greater social isolation and decreased risk of exposure to infection early in an epidemic.

Sickness benefit claims are a well recognized source of epidemiological information and have been used for many years in the surveillance of influenza and the assessment of its impact (McDonald, 1963; Roden, 1963). They have the limitations that only total numbers of claims are currently available and that they apply only to the working population. But, because the scale of sickness absence caused by influenza during epidemics is far greater than that due to any other cause, the total number is a sufficiently sensitive index of influenza prevalence to detect epidemics. As influenza epidemics affect all ages the restriction of information to working persons is no serious handicap, unless, as occurred in 1967–8, a holiday period intervenes when claims for insurance benefit are not submitted: during this period returns from general practice and deaths revealed that the epidemic was continuing to increase at a time when sickness claims showed a sharp temporary drop.

Laboratory reports, though more precise than any other index, are too scanty and based on too selected a population to give a reliable measure of incidence. They do serve, however, to substantiate or refute reports of epidemics and to characterize the virus responsible.

SUMMARY

The occurrence of epidemics of influenza in Britain since 1957, when the A2 (Asian) virus was first introduced, has been traced by an analysis of influenza deaths and sickness insurance benefit claims. In a more detailed study of the epidemic in 1967–8, the largest for 7 years, mortality statistics, sickness benefit claims, consultation rates in general practice and laboratory reports were analysed according to time and geographical location, the age distribution of cases and their clinical features. The value of these various indices in the surveillance of influenza is discussed. It is estimated that the number of cases of influenza during the 1967–8 epidemic was just over one million in the working population and almost two millions in the whole population of Britain.

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