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Gastroenteritis due to *Salmonella* subgenus III (Arizona). A second case diagnosed in Britain

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SUMMARY

The second patient with Arizona gastroenteritis to be diagnosed in Britain had just returned from the United States, where his illness began.

CASE HISTORY

A young Englishman who had lived for 2 years in the U.S.A. spent August, 1970, with his wife hiking and camping in California, Florida and Arizona followed by a 2-day stay in San Francisco. On 2 September 1970, the couple visited the Yellowstone Park where the patient developed quite severe diarrhoea and malaise, but he was too busy packing for return to England to see a doctor until after boarding an east-bound liner on which treatment by the ship's doctor gave him temporary relief. After reaching England the patient stayed for a few days in Winchester, where on 25 September he was seen by one of us (R.E.D.) who gave symptomatic treatment and sent a stool specimen to the laboratory. The patient, who by this time had lost $1\frac{1}{2}$ stones of weight, then left Winchester, but he later reported that by 12 October he was free of symptoms and regaining weight.

We are indebted to the patient himself for a full account of his illness. When he heard that he was suffering from an infection which might have been connected with turtles he remembered that between 17 and 20 August, during a raft trip down the Colorado river, in which turtles occur, he and his wife had drunk river water. The wife remained well.

BACTERIOLOGY

The faecal specimen collected on 25 September was a formed one. It was cultured on deoxycholate citrate agar and on bismuth sulphite agar; an enrichment culture in selenite broth was subcultured on the same two plating media after overnight incubation. The subcultures from selenite broth showed colonies resembling *Salmonella* on both media. Biochemical tests gave the results shown in Table 1.

These reactions identified the organism as a member of the genus *Salmonella*. The strain did not ferment dulcitol, but liquefied gelatin and utilized malonate, reactions which showed that it belonged to *Salmonella* subgenus III, also known as the Arizona group.

Table 1. *Biochemical reactions of the Arizona strain*

Dextrose	Acid and gas	(1)	Citrate (Simmons)	+
Lactose, 1%	−	(7)	Potassium cyanide	−
Salicin	−	(7)	Lysine decarboxylase	+
ONPG	+		Ornithine decarboxylase	+
Dulcitol	−	(7)	Arginine dihydrolase	+
Gelatin liquified			H_2S	+
Malonate utilized				

Figures in parentheses indicate days of incubation of tests.

The Arizona group has its own antigenic scheme with somatic 'O' and flagellar 'H' antigens. Many of these antigens are identical with antigens in the Kauffmann–White antigenic scheme used for salmonellas of subgenera I and II. Using salmonella antisera our strain was shown to have the flagellar 'H' factors z_4, z_{23} and to be monophasic; its 'O' antigens could not be determined with the sera available and it was sent to the Salmonella Reference Laboratory, Colindale, where the antigenic structure was found to be *Arizona* 1,3:1,2,6:−. The equivalent *Salmonella* structure is 44:z_4, z_{23}:−. Edwards, Fife & Ramsay (1959) reported isolations of this serotype from reptiles and from a sample of egg or egg product.

DISCUSSION

Arizona food-poisoning has been more extensively investigated in the U.S.A. than in other parts of the world. In the United States human infections have been reported both as sporadic cases and outbreaks, and in Europe, Asia and Africa sporadic cases have been reported from many areas. In Britain the only previous record of human infection was that of Plows, Fretwell & Parry (1968), who reported gastroenteritis in a girl in Sheffield due to Arizona 26:32:21; another child in the family was a symptomless excreter and the source of infection was shown to have been a pet turtle (*anglice* terrapin) imported from Florida, U.S.A. Thus the only two incidents of human Arizona infection in Britain have both been directly traceable to the U.S.A.

Arizona bacteria have been isolated in Britain from imported bone meal (Harvey & Price, 1962) and from drain swabs in abattoirs where cattle, pigs and sheep were being slaughtered (Harvey, Price & Dixon, 1966). These workers examined 1641 swabs from four different abattoirs but made only six isolations of two Arizona serotypes; therefore in comparison with the *Salmonella* subgenus I serotypes, Arizonas are very rare in British livestock, although the presence of these organisms in animal food might lead to a build-up in the course of time. In the United

States there have been outbreaks in turkeys and chickens but there have been no reports of similar events in Britain. In the U.S.A. and elsewhere reptiles are regarded as reservoirs of Arizona infection, and Brookes & Fife Asbury (1966) reported the isolation of Arizona serotypes from three snakes and a tortoise in the London Zoo.

It has been suggested that Arizona strains are rarely reported from human material in Britain because they are overlooked in British laboratories. The fact that the only two human incidents so far brought to light involved an imported vector and a returning traveller suggests that bacteriologists in Britain are not blind to the possibility of meeting with Arizona strains and that these organisms really are uncommon in Britain. It has been pointed out by some of the authors quoted that the use of a bismuth sulphite medium, such as Wilson and Blair's or de Loureiro's, permits the recognition of Arizona colonies irrespective of lactose fermentation, in contrast to lactose–bile salt media on which lactose-fermenting Arizona strains may be indistinguishable from *Escherichia coli*.

We are grateful to Dr R. Rhode of the National Salmonella Centre, Hamburg, who confirmed the Arizona structure.

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Protective isolation in single-bed rooms: studies in a modified hospital ward

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SUMMARY

Studies were made in a modified hospital ward containing 19 beds, 14 of them in the open ward, one in a window-ventilated side-room, two in rooms with partial-recirculation ventilators giving 7–10 air changes per hour, and two in self-contained isolation suites with plenum ventilation (20 air changes per hour), ultra-violet (UV) barriers at doorways and airlocks.

Preliminary tests with aerosols of tracer bacteria showed that few bacteria entered the plenum or recirculation-ventilated rooms. Bacteria released inside mechanically ventilated cubicles escaped into the corridor, but this transfer was reduced by the presence of an airlock. UV barriers at the entrance to the airlock and the cubicle reduced the transfer of bacteria from cubicle to corridor.

During a period of 4 years while the ward was in use for surgical and gynaecological patients, the incidence of post-operative sepsis and colonization of wounds by multiple-resistant *Staphylococcus aureus* was lower (though not significantly lower) in the plenum-ventilated rooms than in the open ward, the recirculator-ventilated cubicles and the window-ventilated cubicles. Nasal acquisition of multiple-resistant *Staph. aureus* was significantly less common in the plenum-ventilated than in the recirculator-ventilated cubicles and in the other areas. Mean counts of bacteria on settle-plates were significantly lower in the plenum-ventilated cubicles than in the other areas; mean settle-plate counts in the recirculator-ventilated cubicles were significantly lower than in the open ward and in the window-ventilated side-room; similar results were shown by slit-sampling of air. Mean settle-plate counts were significantly lower in all areas when the ward was occupied by female patients. *Staph. aureus* was rarely carried by air from plenum-ventilated or other cubicles to the open ward, or from the open ward to the cubicles; though staphylococci were transferred from one floor area to another, they did not appear to be redispersed into the air in sufficient numbers to infect the patients. Ultra-violet irradiation caused a significant reduction in the total and staphylococcal counts from the floors of airlocks, and a significant reduction of total counts in the air.

INTRODUCTION

Isolation in single-bed rooms has been used for infected patients ('source' or 'containment' isolation), and for the protection of those whose susceptibility to infection is increased by disease or by immuno-suppressive treatment ('protective' isolation). Some evidence of the value of source isolation in ward cubicles has been reported (e.g. Lack, Towers & Stevenson, 1962; Williams *et al.* 1962; Turner, Watson & Abbott, 1965), but there is little published evidence of the value of cubicles for protective isolation. In a burns unit, neither the subdivision of an open ward into cubicles nor the installation of partial recirculation air-conditioners into the cubicles without other aseptic innovations led to any fall in the incidence of burn infections (Cason, Jackson, Lowbury & Ricketts, 1966). Burns offer a particularly severe challenge to any method of protective isolation (Lowbury, 1970), and better results might have been obtained in other types of patient. In a unit where leukaemic patients were treated with cytotoxic drugs, the isolation of patients in specially designed single-bed suites was apparently effective in excluding cross-infection (Robertson *et al.* 1968); but in this environment, as in the bacteria-free ward unit of the new Burn Centre described by Burke (1970), additional aseptic and antiseptic measures (e.g. sterile food or topical chemoprophylaxis) may have provided the main protective barriers against cross-infection.

The studies reported here were made in a ward specially modified to allow an assessment of the value of different types of single-bed rooms and of different types of ventilation as factors in the protective isolation of surgical patients.

Structure of the modified ward

A large 30-bed ward with one single-bed room was modified and equipped with four additional single-bed cubicles (see Fig. 1). Two of these cubicles (described below as plenum-ventilated cubicles) are part of self-contained isolation units,

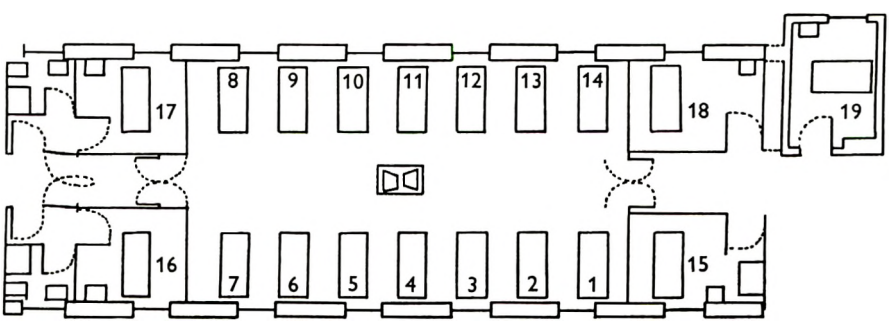

Fig. 1. Plan of ward: 1–14 beds in open ward; 15 and 18 recirculation-ventilated cubicles. 16 and 17 plenum-ventilated cubicles; 19 window-ventilated cubicle.

each having an airlock and an annexe containing toilet, shower and wash-basin. A plenum-ventilation system supplies warmed, humidified and filtered air to both cubicles at a rate of 20 air changes per hour and to the annexes at a rate of

10 changes per hour. The air is passed through a pre-filter which has an efficiency of 93% for 5 μm. particles and a secondary filter which has an efficiency of 99% for 3 μm. particles. There is also a pre-heater, a capillary washer type of humidifier and a fan which has an output of 1100 ft.^{3}/min and drives the air along ducts to the two cubicles and the annexes. A refrigeration system was not included. Ultra-violet strip-lights were fitted over the door of the cubicle and the door separating the airlock from the outside corridor. The UV lights were 30 watt, giving maximum transmission at 2537 Å at an intensity of 83 μW./cm.^{2} at 1 m. The UV lights were only switched on during the experimental periods described.

Two of the other cubicles are equipped with smaller air-conditioning units which recirculate the cubicle air through filters; an additional 20% of air is drawn in from outside. Each cubicle has a different make of air-conditioning plant. A 'West-air Model 300' supplied by Thermotank Limited was fitted in one cubicle (Bed 18, recirculation cubicle 1). The output of the recirculator was approximately 200 ft.^{3}/min. and gave 6–7 air changes per hour in the cubicle; a washable fibre-glass filter with an efficiency of 95% for 5 μm. particle size was fitted. The other cubicle (bed 15, recirculation cubicle 2) was equipped with an air-conditioner supplied by Carter Thermal Engineering Company. The output was 300 ft.^{3}/min. and gave approximately 10 air changes per hour in the cubicle; a disposable fibre filter with an efficiency of 95% for 5 μm. particle size was used with the machine. The units provide warmed air; the 'Westair' machine contains a refrigeration unit but no humidifier, and the 'Carter' unit contains an integral humidifier but no refrigeration unit. The filters of both units showed a reduction of air flow at 6–8 weeks and were changed every month. Neither of these cubicles has airlocks, toilets, or showers. The original single-bed cubicle is furthest from the open ward; it has no air-conditioning unit and is ventilated by windows. Fourteen beds are present in the open section of the ward, giving a total of 19 beds.

PRELIMINARY INVESTIGATION

Before patients were admitted to the ward, tests were made on the efficiency of the isolation units and their ventilation systems with aerosols of *Bacillus subtilis* var. *globigii* or of micrococci used as tracers.

Experiments were made to determine the following:

(1) The rate of clearance of organisms from the cubicles.

(2) The escape of organisms from cubicles to the corridor.

(3) The entrance of organisms into the cubicles when released in the corridor.

(4) The effect of UV barriers on the escape of organisms from a plenum-ventilated unit.

General methods

Tracer organisms

B. subtilis var. *globigii* was grown in nutrient broth for 48 hr. at 37° C. The broth cultures were centrifuged and resuspended in water to give an approximate concentration of 10^9 organisms per ml. The suspension was heated to 100° C. for

10 min. and then kept as the stock suspension at 4° C. In the experiment the stock suspension was diluted 1/100 in nutrient broth containing 0·2% Tween 80.

For the experiments with the UV light barriers, a suspension of a micrococcus was used, since this organism was likely to have a sensitivity to UV irradiation similar to that of *Staphylococcus aureus*. The organism was grown in nutrient broth for 18 hr. at 37° C. The culture was centrifuged and resuspended in nutrient broth containing 0·2% Tween 80 to give a concentration of approximately 10^7 organisms/ml. for the test.

Distribution of organisms

One ml. of the suspension of organisms was dispersed into the air by the use of a spinning disk atomizer (May, 1949). The disk was driven by compressed air at a speed to give aerosol particle sizes of approximately 10–15 μm. over a period of 2 min. During the release of organisms, large hand fans were used to distribute the organisms throughout the room.

Experiments

Clearance of organisms from ventilated cubicles

Aerosols of spores of *B. subtilis* var. *globigii* were released in the cubicles by the technique described and the air was sampled on nutrient agar plates with a slit-sampler over a period of 30 min.; viable counts were made after incubation of the plates for 18 hr. at 37° C. Tests were made in one of the plenum-ventilated cubicles and in the two recirculation-ventilated cubicles; replicate tests were made with the ventilation switched off, and with the ventilation switched on during and after the release of the organisms.

Table 1. *Clearance of* B. subtilis *var.* globigii *from ventilated cubicles*

Time after release (min.)	Cubicle	Number of *B. subtilis* var. *globigii* in 12 ft.³ of air: Ventilation switched on after release of organisms	Ventilation on throughout	Ventilation switched off
1	Plenum	267	70	150
5		101	35	156
10		20	11	146
20		2	2	119
30		1	1	80
1	Recirc. 1	260	130	150
5		168	94	110
10		120	65	91
20		60	31	82
30		30	20	80
1	Recirc. 2	378	317	185
5		245	160	189
10		189	106	160
20		80	50	141
30		48	8	121

Results. These are shown in Table 1. Clearance of *B. subtilis* var. *globigii* occurred more rapidly and completely in all cubicles when the ventilation was switched on. A lower peak count and more rapid clearance were obtained if ventilation was on during the release of organisms than if the ventilation was switched on after the release of the organisms. A more rapid and complete clearance was obtained in the plenum-ventilated cubicles than in the other two cubicles. This corresponded to the higher rate of flow in the plenum-ventilated than in the recirculation-ventilated cubicles.

The transfer of organisms released inside cubicles to the corridor outside

Aerosols of *B. subtilis* var. *globigii* spores were released in a plenum-ventilated cubicle. Simultaneous samples were obtained on nutrient agar plates with one slit-sampler inside the cubicle and another slit-sampler placed in the corridor immedi-

Table 2. *Transfer of* B. subtilis *var.* globigii *from a plenum-ventilated cubicle to an airlock or to outside corridor*

Time after release of organism (min.)	Number of *B. subtilis* var. *globigii* in 12 ft.³ of air			
	Inside cubicle	In corridor outside cubicle	Inside cubicle	In airlock
1	292	8	265	115
2	318	20	230	160
	Doors opened*		Inner door opened (30 sec.)	
3	220	32	152	144
4	133	18	120	119
5	120	12	68	84

* The inner door was opened for 30 sec., closed and outer door was opened for 30 sec.

Table 3. *Transfer of* B. subtilis *var.* globigii *from ventilated cubicle (recirculation 1) to corridor*

Time after release of organism (min.)	Number of *B. subtilis* var. *globigii* in 12 ft.³ of air			
	Inside cubicle		In corridor outside cubicle	
	Vent off	Vent on	Vent off	Vent on
1	240	208	5	42
2	260	140	2	38
		Door opened (30 sec.)		
3	222	120	50	72
4	184	109	23	68
5	160	80	20	62

ately outside the door of the unit. To simulate a person leaving the unit, the inner door of the airlock was opened for 30 sec., 2 min. after the release of the organisms; the inner door was then closed and the outer door was opened for 30 sec. The experiment was repeated with the second slit-sampler placed in the airlock and the inner door only was opened for 30 sec. at 2 min. Spores were similarly released in

one of the cubicles ventilated with a recirculation unit. Samples were collected with one slit-sampler inside the cubicle and the other outside the door of the cubicle. The door was opened at 2 min. for 30 sec. Separate experiments were made with the ventilation switched on and off.

Results. Table 2 shows that some organisms escaped from the cubicle through the airlock into the corridor. The numbers of organisms increased slightly when the doors were opened. Many more organisms were obtained from the airlock than from the outside corridor. Table 3 shows that when the ventilation was off in the recirculation cubicle, few organisms escaped until the door was opened. With the ventilation on, more organisms escaped with the door closed and numbers increased when the door was opened. These experiments demonstrate that there is a risk of airborne organisms escaping from ventilated cubicles, but this may be reduced by the presence of an airlock.

The transfer of organisms from outside to inside of cubicles

An aerosol of *B. subtilis* var. *globigii* spores was released in the corridor outside the plenum-ventilated cubicle. Simultaneous air samples were taken outside and inside the cubicle. Separate experiments were made with the ventilation switched on and off. The doors were opened for 30 sec. at 2 min. A similar experiment was made with one of the recirculation-ventilated cubicles.

Table 4. *Transfer of* B. subtilis *var.* globigii *from corridor outside a plenum-ventilated cubicle with airlock to inside of cubicle*

Time after release of spores (min.)	Number of *B. subtillis* var. *globigii* in 12 ft.³ of air			
	Inside cubicle		Outside cubicle	
	Ventilation off	Ventilation on	Ventilation off	Ventilation on
1	1	0	174	145
2	3	4	160	122
	Doors opened for 30 sec.			
3	7	8	155	100
4	11	5	149	75
5	16	4	104	53
6	26	1	68	28
7	22	0	72	22
8	21	0	52	21
9	20	3	35	12
10	21	1	37	14

Results. Tables 4 and 5 show that few organisms entered either the plenum-ventilated or recirculation-ventilated cubicles with the ventilation system switched on even when the doors were opened as in the previous experiment. More organisms entered when the doors were opened and the ventilation was switched off. The experiments demonstrate that few airborne organisms are likely to enter the ventilated cubicles, particularly the plenum-ventilated cubicle with the ventilation on.

Table 5. *Transfer of* B. subtilis *var.* globigii *from corridor outside ventilated cubicle (recirculation* 1*) to inside*

Time after release of spores (min.)	Number of *B. subtilis* var. *globigii* in 12 ft.³ of air			
	Inside cubicle		Outside cubicle	
	Ventilation off	Ventilation on	Ventilation off	Ventilation on
1	0	1	400	500
2	1	2	200	300
	Door opened for 30 sec.			
3	0	1	180	242
4	12	4	132	202
5	8	3	140	184
6	8	8	159	105
7	5	6	143	132
8	12	5	148	120
9	9	1	112	80
10	14	3	75	75

Table 6. *The effect of UV barriers on the escape of organisms from a plenum-ventilated cubicle*

Time of Sampling	Doors	Total micrococci in 12 ft.³ of air			
		Inside cubicle		Corridor outside cubicle	
		UV off	UV on	UV off	UV on
Before release of organisms	Closed	21	10	4	13
2 min. after release		532	497	17	15
4 min. after release		461	534	17	14
6 min. after release		231	284	39	13
8 min. after release		212	270	30	11
Before release of organisms	Open	13	10	14	58
2 min. after release		370	293	484	153
4 min. after release		114	76	275	73
6 min. after release		50	46	103	57
8 min. after release		41	31	84	34

The effect of UV light barriers on the escape of organisms from a plenum-ventilated cubicle

An aerosol of micrococci was released in one of the plenum-ventilated cubicles. Simultaneous air samples were collected with one slit-sampler in the cubicle and another in the corridor outside the airlock. The experiment was repeated with the UV light barriers switched on. Similar experiments were made with the doors closed and with the doors open.

Results. Table 6 shows that larger numbers of organisms escaped from the cubicle during the period of sampling when the UV was off than when it was switched on.

When both doors were opened with the ventilation on and the UV barriers off, more organisms were obtained outside the cubicle than within. The number of organisms outside the cubicle was less when the UV barriers were switched on. The results demonstrate that two UV light barriers irradiating the airlock cause some reduction in the numbers of organisms escaping from the cubicle.

CLINICAL STUDY

The ward was studied from October 1965 to December 1969, apart from several periods during which it was either closed or used for patients whom we did not include in our series. A total of 1674 patients were included in the study. The type of patient was changed at approximately yearly intervals throughout the period; female surgical and gynaecological patients were admitted for 2 years and male surgical patients for a rather shorter period. Patients who were likely to remain in hospital for 10 days or longer were randomly selected for admission to the isolation cubicles and to the open ward; those admitted to cubicles remained in them throughout their stay, although during the later stages of the investigation a few patients were admitted either for special protection or because they were staphylococcal dispersers. Occasionally patients refused to remain in the cubicles and were transferred to the open ward.

The ward was used mainly as an ordinary surgical ward, but during the first 2 years few patients were admitted for emergency surgery. Beds in the open ward were not moved unless there was a medical indication, which was rare. Patients in side-rooms were treated as other patients and no special precautions were taken (e.g. the use of mask, gowns, overshoes, and special hand-washing techniques) except for a few patients requiring protective isolation. Visiting was not restricted. Patients in the self-contained plenum-ventilated units remained in them throughout their stay in hospital, but patients in the recirculation and window-ventilated rooms walked through the open ward to bathroom and w.c.'s. Information on all patients admitted to the ward was entered on special record cards. After the first year the information was transferred to forms suitable for transfer to punch cards. In addition to personal details the information included bed-site in ward, days in hospital, diagnosis, operation, wound and other sepsis, factors predisposing to infection, antibiotics given and bacteriological results. All ward records were examined when the patient was discharged and information on sepsis was obtained

daily from the ward sister by the laboratory staff and entered in a day book. Wounds were classified as septic if there was any evidence of clinical infection irrespective of the presence or absence of pus. Swabs were taken daily for 6 days a week from patients' noses and weekly from the staff. Nose swabs were taken from patients on admission by the staff at weekends and placed in Stuart's transport medium. Swabs were taken from drained or moist wounds at the first dressing and at subsequent dressings whenever possible, also from any other infected lesions. Settle-plates (see below) were exposed for 2 hr. on 5 days a week; two plates were exposed in each side-ward and seven in the open ward. The plates were placed on bed-side lockers and bed tables, approximately 3 ft. above the floor. Slit-sampling studies were also made twice weekly. This routine was continued throughout most of the period, but the frequency of sampling was reduced for limited periods because of other laboratory commitments. Counts of bacteria on floors were made, initially with gauze-impregnated agar (Foster, 1960) and later with Alne plastic contact plates (Hall & Hartnett, 1964), when information on floor contamination was required.

Bacteriology

Nasal swabs were cultured on nutrient agar containing 1% serum and phenolphthalein diphosphate (Barber & Kuper, 1951). The same medium was used for settle-plates, surface sampling plates and slit-sampling studies. Counts of total organisms and presumptive *Staph. aureus* were made after 18 hr. incubation at 37° C. Either four colonies or 10% of colonies of presumptive *Staph. aureus* (whichever was the higher) were confirmed by slide or tube coagulase tests. Wound swabs were cultured aerobically on blood agar, McConkey medium and in cooked meat broth, which was subcultured aerobically and anaerobically at 48 hr. Tests of antibiotic sensitivity on *Staph. aureus* were made by a ditch plate method (Topley, Lowbury & Hurst, 1951). Antibiotic sensitivity tests were made on strains grown from the environment and the first strain grown from a nose and wound and then at weekly intervals. If a change in sensitivity was noted, strains collected during the previous week also were examined. Phage typing (Blair & Williams, 1961) was carried out on environmental strains resistant to at least two antibiotics, on the first strains isolated from nose or wound and again subsequently if there was a change in antibiotic resistance.

Results

Comparability of groups of patients

Patients in the open ward and cubicles were comparable both in their length of stay and in numbers receiving antibiotics. Patients admitted during 1965, totalling 433, were excluded from this comparison, since information on their length of stay and administration of antibiotics was not available for that period. In an analysis of 1241 patients admitted between 1966 and 1969 the average length of stay of patients was 10·9 days in the open ward, 10·7 days in the plenum-ventilated and recirculator-ventilated cubicles and 9·3 days in the window-ventilated cubicle. Thirty-four per cent of patients in the open ward and recirculator-ventilated cubicles, 31% in the plenum-ventilated cubicles and 32% in the window-ventilated cubicle were treated with antibiotics.

Wound sepsis and staphylococcal infection

The overall incidence of wound sepsis in the cubicles and the open ward from 1966 to 1969 was 62/744 (8·3%); the incidence was 41/610 (6·7%) in undrained wounds and 21/134 (15·7%) in drained wounds.

The incidence of wound sepsis was lower in the plenum-ventilated wards (2/68 = 2·9%) than in the open ward (53/596 = 8·9%), the recirculator-ventilated cubicles (4/52 = 7·7%) or the window-ventilated cubicle (3/28 = 10·7%). However, the number of wounds in which there was a special hazard of infection in the wards after operation was too small for comparison of cubicles with the open ward; e.g. the incidence of sepsis in drained wounds was 15/104 in the open ward, 3/12 in the recirculator-ventilated cubicles and 1/12 in the plenum-ventilated cubicles. Table 7 shows the incidence of staphylococcal infection from 1965 to 1969 and includes colonized as well as septic wounds. Multiple-resistant strains (i.e. resistant to two

Table 7. *Colonization of wounds with* Staph. aureus *1965–69*

Site	Incidence of colonization with multiple-resistant strains	Incidence of colonization with sensitive or penicillin-resistant only strains
Open ward	16/722 (2·2%)	20/722 (2·8%)
Recirculation cubicles	2/61 (3·3%)	1/61 (1·6%)
Plenum cubicles	0/78	3/78 (3·8%)
Window-ventilated cubicle	1/26 (3·8%)	0/26

Table 8. *Acquisition of multiple-resistant* Staph. aureus *in noses (1965–69)*

Site	Total patients	Number of acquisitions
Open ward	1325	51 (3·8%)
Recirculation cubicles	134	8 (5·9%)
Plenum-ventilated cubicles	143	1 (0·7%)
Window-ventilated side-ward	72	3 (4·2%)
Significant differences: comparison of:		
Plenum with open ward		$t = 1{\cdot}94$ $P \simeq 0{\cdot}05$
Plenum with recirculation cubicle		$t = 2{\cdot}47$ $P < 0{\cdot}05$

or more antibiotics) are likely to have been acquired in the ward, since patients usually had operations on the day after admission and theatre staff infrequently carried multiple-resistant strains. There were no infections (0/78) with these strains in the plenum-ventilated cubicles but this incidence was not significantly lower than in any of the other areas.

Nasal acquisition of multiple-resistant Staph. aureus

Isolation of multiple-resistant strains from noses of patients who carried sensitive, penicillin-resistant strains only or no *Staph. aureus* on admission were considered to be acquisitions in the ward. The acquisition of multiple-resistant *Staph. aureus* is shown in Table 8. The number acquiring strains in the plenum-ventilated cubicle was less than in the open ward and also less than in the recirculation cubicles,

both differences being significant. The other differences are not significant; there was no significant difference in the frequency of acquisition of multiple-resitant strains in the recirculation-ventilated cubicles, open ward and non-ventilated side-ward.

Airborne organisms

The mean counts of total organisms on settle-plates over a period of 2 years are shown in Table 9. The ward was occupied by female patients from October 1965 to September 1966 and by male patients from October 1966 to September 1967; there

Table 9. *Settle-plate counts*

Period of study	Sex of patients in ward	Site of sampling	Number of plates	Mean/total count/plate /h. and S.E.
October 1965 to September 1966	Female	Window-ventilated cubicle	262	10·8 ± 0·90
		Recirculation cubicles	726	8·8 ± 0·41
		Open ward	1421	14·5 ± 0·27
		Plenum-ventilated cubicles	569	3·9 ± 0·25
October 1966 to September 1967	Male	Window-ventilated cubicle	295	23·6 ± 1·45
		Recirculation cubicles	752	15·9 ± 0·64
		Open ward	1568	26·0 ± 0·50
		Plenum-ventilated cubicles	563	12·6 ± 0·59

Comparison of:	Female		Male	
Plenum with open ward	$t = 28{\cdot}60$	$P < 0{\cdot}001$	$t = 17{\cdot}32$	$P < 0{\cdot}001$
Plenum with recirculation cubicle	$t = 10{\cdot}08$		$t = 3{\cdot}79$	
Plenum with window-ventilated cubicle	$t = 7{\cdot}34$		$t = 7{\cdot}01$	
Recirculation with open ward	$t = 11{\cdot}49$		$t = 12{\cdot}46$	
Recirculation with window-ventilated cubicle	$t = 2{\cdot}01$	$P < 0{\cdot}05$	$t = 4{\cdot}85$	
Window-ventilated cubicle with open ward	$t = 6{\cdot}03$	$P < 0{\cdot}001$	$t = 1{\cdot}57$	Not significant

was considerable variation in counts on individual plates and on single days. For both years the results obtained showed the same order, counts in the open ward being highest and counts in the plenum-ventilated cubicle being lowest. The differences between all but two of these mean counts for both years showed highly significant results. For the males only the difference between the window-ventilated cubicle and the open ward was not significant. The difference between the window-ventilated and recirculation-ventilated cubicles for females was just significant ($P < 0{\cdot}05$). All the other results were highly significant at the level $P < 0{\cdot}001$. The counts in the open ward and cubicles were twice as high during the period of male occupation as during the female; the difference was highly significant in all areas ($P < 0{\cdot}001$).

The slit-sampling studies showed similar differences; e.g. from October to

December 1967 the total mean count was 8·1 per ft.^{3} for the window-ventilated cubicle, 6·5 per ft.^{3} for the recirculation cubicles, 3·5 per ft.^{3} for the plenum-ventilated cubicles and 12·4 per ft.^{3} for the open ward.

Transfer of Staph. aureus *in the air to and from cubicles*

The assessment of this was difficult owing to the small number of dispersers and because more than one patient in the ward at one time carried a strain of similar phage and antibiotic sensitivity pattern. *Staph. aureus* was usually present on settle-plates or on slit-sampling plates in small numbers and there was little evidence of transfer either from cubicles to the open ward or vice versa.

The presence of a few staphylococcal dispersers enabled some observations to be made. On one occasion, when a disperser was present in a plenum-ventilated cubicle, 89 colonies were isolated on settle-plates in the cubicle over a period of 6 days; 10 colonies were phage typed and showed a similar phage pattern and only three colonies of similar type were isolated in the main ward. Slit-sampling counts on another day showed 13 colonies of *Staph. aureus* in the cubicle and none in the corridor or in the other plenum-ventilated cubicle. The evidence suggests that few organisms escaped from the plenum-ventilated cubicle into the open ward.

When a disperser of a strain of *Staph. aureus*, phage type 84/85, was in a recirculation cubicle, 258 colonies of *Staph. aureus* were grown on settle plates, each exposed for 2 hr., during a 3-day period; 77 colonies of *Staph. aureus* were grown in the main ward, and of 16 colonies typed, 9 were of the same type as those in the recirculation cubicle. Two colonies of similar type were grown from the other recirculation cubicle and none from the plenum-ventilated cubicles.

On another occasion, when smaller numbers of *Staph. aureus* were grown from a recirculation cubicle (23 colonies) on 1 day, only one colony of similar type was grown from settle-plates in the open ward. At another time when 42 colonies were grown from settle-plates in the window-ventilated cubicle, only 1 colony of similar type was grown in the main ward on the same day. The relative absence of airborne transfer from the main ward to the cubicles is shown in the following examples: (1) 27 colonies of *Staph. aureus* grown on slit-sampling plates in the open ward and none in any of the cubicles; (2) 65 colonies in the open ward and one colony in one of the plenum-ventilated cubicles; (3) 22 colonies in the open ward and none in the cubicles.

The results confirm an interpretation of the original studies with artificially released organisms. Few staphylococci entered the cubicles by air from the main ward, but when the level of contamination was high, organisms did escape from the recirculation cubicle into the open ward, possibly because of the absence of an airlock. The window-ventilated cubicle was further than the other cubicles from the open ward and sampling showed that organisms were less likely to be transmitted from it in the air to the open ward.

Distribution of Staph. aureus *on floors*

Heavy floor contamination of the open ward was often associated with contamination of the floors of cubicles with the same phage type of *Staph. aureus.*

On one occasion 19/20 contact plates taken from the main ward showed *Staph. aureus* resistant to penicillin, tetracycline and erythromycin of phage type 80/81 (at R.T.D.). The same strain was found on 3/10 contact plates taken in a recirculation cubicle and on 6/10 plates taken in a plenum-ventilated cubicle. Mean counts of *Staph. aureus* were 12·8/plate in the open ward, 2·5/plate in the recirculation cubicle and 2·0/plate in the plenum-ventilated cubicle. One colony was grown on settle-plates in the recirculation cubicle and none in the plenum-ventilated cubicle. Similar results were obtained on several other occasions when floor contamination of the open ward was heavy. Patients in the cubicles did not acquire the strain in the nose or wound. When floor contamination was light, staphylococci of the same phage type were rarely found in the cubicles. The results suggest that although organisms were transferred from one floor area to another, probably on the soles of shoes, they were not transferred from the floor to the air in sufficient numbers to infect or colonize the patients.

UV light barriers

The effect of the two UV light barriers on bacteria in one of the airlocks was investigated when the plenum-ventilated cubicles were occupied by patients. In one series of experiments a comparison was made between the two cubicles and

Table 10. *The effect of UV light barriers in an air lock*

Slit-sampling studies in plenum-ventilated units.

Site of sampling	Mean bacterial counts in 50 ft.³/air					
	UV on			UV off		
	No. of plates	Total organisms	Total *S. aureus*	No. of plates	Total organisms	Total *S. aureus*
Cubicle	16	434·6 ± 121·7	6·3	13	309 ± 103·9	2·8
Airlock	16	120 ± 19·9	0·4	13	250·6 ± 35·3	1·5

their airlocks; the UV lights were switched on in one airlock but not in the other. In the other series, the same cubicle and airlock were investigated, both with and without UV irradiation of the airlock. UV lights were switched on at least 24 hr. before each set of observations. The air of the cubicles and airlocks was sampled with a slit-sampler and floors were examined with 8–10 contact plates for each experiment. Bacteriological techniques were as described in the general methods.

The overall results of the slit-sampling studies are shown in Table 10. Total counts of organisms were significantly lower ($t = 3{\cdot}3$, $P = < 0{\cdot}01$) and *Staph. aureus* were lower (but not significantly so) in the irradiated than in the unirradiated airlock; mean counts were lower in the airlocks than in the cubicles by 75% with the UV irradiation on, but by only 19% with the UV off. In the airlock with the UV off 9/13 bacterial counts were higher than in the corresponding cubicle, but only 2/16 airlock counts were higher than the cubicle counts when the UV lights were switched on. Table 11 shows that counts of total organisms and *Staph. aureus* on the floor of the airlock were significantly reduced (total organisms: $t = 9{\cdot}57$, $P < 0{\cdot}001$) when irradiated with UV lights.

Table 11. *The effect of UV light barriers in an airlock*

Contact plates from floors of plenum-ventilated units

Site of sampling	Mean bacterial count/plate					
	UV on			UV off		
	No. of plates	Total organisms	Total *S. aureus*	No. of plates	Total organisms	Total *S. aureus*
Cubicle	71	106·8 ± 18·8	3·6	68	269·9 ± 28·1	3·5
Airlock	81	12·8 ± 2·2	0·02	75	190·8 ± 19·1	3·6

DISCUSSION

In these studies on a modified hospital ward we have examined the role of structural separation of patients, with or without the additional aid of mechanical-ventilation and UV barriers, as a component of protective isolation; isolation in a cubicle was assessed in patients who were not also receiving protection by other means, e.g. gowns, masks or a special hand-washing routine.

Preliminary studies with tracer organisms showed that the movement of bacteria between the isolation rooms (cubicles) and the corridor was reduced by mechanical-ventilation, that the presence of an airlock added to this control of bacterial movement, and that UV irradiation of the airlock, whether plenum-ventilation was in use or not, caused a further reduction in the numbers of airborne bacteria which escaped from the cubicle to the corridor. The results of floor sampling obtained in the experiments on UV irradiation with a patient in the cubicle should be interpreted with caution, since organisms on the floor of the airlock are unlikely to be relevant in the spread of infection. The reduction in the count of airborne bacteria in the irradiated airlock suggests that UV barriers might be useful where plenum-ventilation is not used, but in a ward with little contamination, plenum-ventilation alone was effective in preventing airborne organisms from entering from outside. Multiple-resistant strains of *Staph. aureus* were frequently grown from the floor of the cubicles when contamination of the main ward with those organisms was heavy, probably transferred on the shoes of the staff. The absence of colonization of wounds or staphylococcal sepsis with these strains in patients in the cubicles and the absence of these organisms in the air confirm experiments previously reported that organisms on the floor were not easily redistributed into the air (Ayliffe *et al.* 1967).

The mean counts of total airborne bacteria were reduced in proportion to the number of air changes, i.e. settle-plate and slit-sampling counts were lowest in the plenum-ventilated cubicles, higher in the recirculation-ventilated rooms and highest in the open ward or window-ventilated cubicle. There were considerable day-to-day variations in counts in all areas and occasionally counts in one of the cubicles would be unusually high, presumably due to the presence of a heavy disperser of coagulase negative staphylococci. Counts in all areas were consistently higher when the ward was occupied by male patients than when it was occupied by females; it seems likely that males disseminate more organisms into the environ-

ment. This applies also to dispersal of *Staph. aureus* (Bethune, Blowers, Parker & Pask, 1965; Emslie, 1966) and has been confirmed by other observations of our own. A comparison of the total numbers of *Staph. aureus* in the open ward and in the cubicles is not shown, since their presence in the air of a cubicle was almost always related to the presence of a carrier or infected patient in the cubicle; some nasal carriers were always present in the open ward.

In addition to the rarity of *Staph. aureus* in the air of the plenum-ventilated cubicles when no carriers or infected patients were present, there was evidence of protection against airborne spread in the significantly lower nasal acquisition rate of multiple-resistant *Staph. aureus* in patients nursed in these cubicles, when compared with patients nursed in the open ward or in other cubicles. Multiple-resistant strains were used as an index of acquisition since penicillin-resistant strains are now frequently found in the general population and the frequency of acquisition of these strains cannot be assessed with accuracy. The colonization of wounds was also less common (though not significantly so) in patients nursed in the plenum-ventilated cubicles than in those nursed in the open ward or in the other cubicles. The apparent ineffectiveness of the recirculator cubicles in preventing airborne spread was at first sight surprising, in view of the encouraging results obtained in the preliminary bacteriological experiments. It was, however, necessary for these patients (unlike those in the plenum rooms) to walk through the open ward to reach the communal bathroom and w.c., and it is likely that they acquired staphylococci during such excursions into another ward area. The number of multiple-resistant *Staph. aureus* in the open ward was small during most of the period of study, and there was little evidence of cross-infection. The nasal acquisition rate in 1967 was 12/410 (2·9%), whereas in another male surgical ward in the same hospital it was 59/419 (14%) during a 6-month period of the same year. The reason for this difference is not clear, but the smaller number of patients (14 in this ward, compared with 30 in other wards), better bed-spacing, the dilution of surgical with short-stay gynaecological patients when the ward was occupied by female patients, and the high proportion of clean surgery in relatively fit patients admitted from home, may all have contributed to the lower incidence. Since most of the wounds were not drained, the number at risk of acquiring infection in the wards was small; some of the septic drained wounds may have acquired infection in the operating theatre or sepsis may have been due to self-infection by the patient's own flora. Although the wound sepsis rate was lower in the plenum-ventilated cubicles, the numbers were too small for a valid comparison to be made between the cubicles and the open ward.

This study shows that cubicles with plenum-ventilation at 20 air changes per hour, each with associated w.c. and shower and with an airlock, give effective protection against airborne staphylococci in a clean surgical ward. Division of a ward into smaller units, with or without mechanical ventilation, has been associated in some hospitals with a reduced incidence of staphylococcal infection (Edmunds 1970; Smylie, Davidson, Macdonald & Smith, 1971; Davidson, Smylie, Macdonald & Smith, 1971); in other hospitals, however, these conversions have not led to a reduced acquisition of staphylococci and less infection (Whyte, Howie &

Eakin, 1969; Lidwell *et al.* 1970; Cason *et al.* 1966). The use of plastic ventilated isolators which kept out both airborne and personnel-transmitted contaminants achieved only a marginal protection against the acquisition of multiple-resistant *Staph. aureus* in a burns unit (Lowbury, Babb & Ford, 1971); there, however, patients who were more liable to colonization by staphylococci were also exposed to a greater challenge of contamination than in a clean surgical ward. *Pseudomonas aeruginosa* and some other Gram negative bacilli, which are of special importance as pathogens in patients with severe burns or under treatment by immunosuppressive drugs, appear to be transferred almost entirely by contact except at the time of removal of heavily contaminated dressings; isolation techniques aimed at excluding airborne transfer of bacteria cannot be expected to affect the incidence of infection with these bacteria, except in rooms where dressings of burns are changed (Lowbury, 1954; Lowbury *et al.* 1971).

A single-bed room without mechanical-ventilation, though ineffective in controlling cross-infection in a burns unit, may provide adequate structural isolation for most patients requiring special protection, particularly if sited in a gynaecological ward or in other wards where the staphylococcal infection rate is low; though airborne transfer of bacteria to patients in such rooms is not prevented, the numbers of bacteria in unoccupied, window-ventilated rooms are much smaller than those in the open ward, even if the door is left open (Lowbury *et al.* 1971). Where airborne contamination is low, barriers against contact transfer are more important and may be sufficient to exclude most of the pathogens. If it is particularly important to prevent airborne infection (e.g. in an isolation room near a ward where there is much staphylococcal contamination) it is possible to give useful protection in an ordinary side-ward equipped with recirculating-ventilators giving a large turnover of air and a small irradiated airlock, such as that which was found effective in preventing airborne spread of bacteria to kidney transplant patients at the Hammersmith Hospital (Ayliffe, 1963). A window-ventilated room with an airlock, irradiated with UV or equipped with an extractor fan, is a possible and even less expensive alternative. The expensive and elaborate self-contained, plenum-ventilated isolation suite would be appropriate for restricted use in the treatment of some 'high risk' patients (e.g. those treated with cytotoxic drugs) for whom sterile supplies of food and of other materials are also required to ensure the absolute minimum of microbial contamination; such patients, however, are still exposed to the risks of endogenous infection with micro-organisms colonizing the intestinal tract, the skin and other surfaces.

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Protective isolation in a burns unit: the use of plastic isolators and air curtains

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SUMMARY

The use of plastic isolators and of an 'air curtain' isolator for protection of patients against infection was studied in a burns unit.

Preliminary bacteriological tests showed that very few airborne bacteria gained access to a plastic ventilated isolator; even when the filter and pre-filter were removed from the air inflow, settle-plate counts inside the isolator were much lower than those in the open ward, but the difference was smaller in tests made with an Anderson air sampler, which showed also that fewer large bacteria-carrying particles appeared inside the isolator than outside it. An open-topped isolator allowed virtually free access of bacteria from ambient air. The numbers of airborne bacteria inside an air curtain were appreciably lower than the counts of airborne bacteria in the open ward, but not as low as those in the plastic ventilated isolator.

Controlled trials of isolators were made on patients with fresh burns of 4–30% of the body surface; the patients were given no topical chemoprophylaxis against *Staphylococcus aureus* or Gram-negative bacilli. Patients treated in plastic isolators showed a significantly lower incidence of infection with *Pseudomonas aeruginosa* than those treated in the open ward; this protective effect was shown by isolators with or without filters or with an open top. Ventilated isolators, which protected patients against personal contact and airborne infection, gave a limited protection against multi-resistant 'hospital' strains of *Staph. aureus*, but no such protection was given by an open-topped isolator, which protected only against personal contact infection, or by air curtains, which protected only against airborne infection; the air curtain gave no protection against *Ps. aeruginosa*, and there was no evidence of protection by any isolator against *Proteus* spp. and coliform bacilli.

Both the controlled trials and evidence from the bacteriology of air, hands, fomites and rectal and nasal swabs taken on admission and later, supported the view that *Ps. aeruginosa* is transferred mainly by personal contact, *Staph. aureus* probably by air as well as by contact and coliform bacilli mainly by self infection with faecal flora, many of which are first acquired from the hospital environment in food or on fomites.

The use of plastic isolators is cumbersome, and of limited value except in the control of infection with *Ps. aeruginosa*. For this reason and because of the effectiveness of topical chemoprophylaxis such isolators are unlikely to have more

than an occasional use in the treatment of burns. Though air curtains greatly reduce airborne contamination, their use in a burns unit does not appear to protect patients against infection when the alternative (and, for *Ps. aeruginosa*, more important) routes of contamination by personal contact and fomites are left open.

INTRODUCTION

Patients with uninfected burns are commonly assumed to require protective isolation in hospital (e.g. Colebrook, 1950; U.S. Public Health Service, 1970). In this hospital the subdivision of an open ward into cubicles and the subsequent installation of air conditioning units in the cubicles did not lead to any fall in the incidence of infection with *Streptococcus pyogenes*, *Staphylococcus aureus* or *Pseudomonas aeruginosa* (Cason, Jackson, Lowbury & Ricketts, 1966). It appeared that the protection given by such structural barriers was insufficient, and that a more effective system should be sought. One method which would be expected to give better protection was the use of plastic isolators. This type of equipment, originally developed for the study of germ-free animals (Reyniers & Trexler, 1943), has been adapted for use in the treatment of burns by Levenson, Trexler, La Conte & Pulaski (1964) and Levenson *et al.* (1966) and by Haynes & Hench (1966); it offers protection against bacterial contamination transferred both by contact and by air. Another method, which offers protection only against airborne contaminants, is the use of special systems of ventilation, such as unidirectional ('laminar') air flow (Lidwell & Towers, 1969) and of air curtains surrounding the patient's bed.

In the studies reported here we have examined the value of plastic isolators and of air curtains in the treatment of freshly burned patients. Controlled trials were made to assess the frequency of infection with the common pathogens of burns. The relative importance of airborne and of personal contact transfer was studied in a comparison of isolators which gave protection against one or the other of these routes, or against both of them.

THE ISOLATORS

Plastic isolators

The Vickers patient isolator was adopted for study after preliminary investigation of some other systems, including an isolator made of rigid plastic. The Vickers isolator (Model 55) consists of a 'canopy' of transparent, flexible plastic (polyvinyl chloride) which is suspended on a metal framework attached to the bed (see Pl. 1, fig. 1); when closed and inflated the canopy completely envelops the patient and rests on the mattress. On each side of the canopy are five glove ports for aseptic handling of the patient, and a pouch with inner and outer zip fasteners, the inner one being opened and closed from the inner aspect through a glove port; one pouch is for the supply of clean or sterile materials to the patient, the other for removal of discarded and contaminated objects.

To admit a patient, the canopy is opened by a zip fastener which runs along the upper surface from one end to the other. When this is closed, the canopy is inflated

and ventilated with air pumped from the ward by a quiet centrifugal fan unit through a coarse pre-filter of spun nylon, to trap the larger dust particles, and a main filter of glass paper with an efficiency of more than 99·9% against particles down to 0·58 μm. in diameter. The coarse filters were changed weekly; the fine filter was changed after 12 months use. The fan unit delivered air at approximately 40 ft.^{3} per min.

During the study many improvements were made in the design of the isolators, based on observations of their use in the treatment of patients with burns. Special modifications included a ventilated half-suit to allow better access by the nurses to all parts of the enclosure. In one of the trials an isolator was used without a main filter, in another both main filter and pre-filter were removed. For the last trial in this series an open-topped canopy was used (Pl. 1, fig. 2), providing free circulation of air to the patient; this isolator protected the patient only against personal (especially manual) contact transfer of bacterial contaminants.

Air curtains

The 'Sterair' Patient Isolator (W.H.S. Pathfinder Ltd.) was used to provide air curtains around the patient's bed; its appearance and mode of action are shown in Pl. 2, figs. 3 and 4. Air is pumped from the open ward by a quiet fan unit in the console at the head of the bed through coarse pre-filters, one on each side of the console, and then through a main filter. The horizontal canopy above the bed has parallel linear apertures on its lower surface, from which air sweeps downwards at a low velocity over the bed, and downwards and outwards from a peripheral aperture at a higher velocity around the bed, with a total turnover of about 1200 ft.^{3} per min.; the peripheral air flow acts as the air curtain. The efficiency of the pre-filters (woven cotton) or glass fibre is stated to be 98% on particles of 5–10 μm., and that of the main filter (glass fibre) to be more than 99·9% for particles of 0·3 μm. The coarse filters were checked by daily tests with an anemometer, and when the air flow rate began to fall a new filter was inserted; such replacement was usually needed every 3 or 4 weeks.

BACTERIOLOGICAL STUDY ON ISOLATORS

Tests were made in empty isolators to assess the degree of protection they provided against contamination with airborne bacteria.

Plastic ventilated isolators

Groups of 6–12 settle plates containing phenolphthalein diphosphate agar (Barber & Kuper, 1951) were exposed for 6 hr. on the bed in the isolator and on tables at about the same level outside the isolator. The plates were incubated at 37° C. overnight, and the total numbers of colonies were counted. Viable counts of airborne bacteria inside and outside the isolator were made on phenolphthalein diphosphate agar plates exposed in an Anderson sampler, from which the bacteria-carrying particle-size distribution could also be assessed. In some experiments

presumptive *Staph. aureus* colonies were counted (i.e. colonies of staphylococcal type giving a positive phosphatase reaction after exposure to ammonia vapour).

Separate tests were made on isolators provided with coarse and fine filters, with coarse filters only and with no filters.

Results

The results are shown in Tables 1 and 2. Mean settle-plate counts obtained outside the isolator were 46·7, compared with mean counts of 0·1 in an isolator with filter and pre-filter and 1·0 in an isolator with neither filter nor pre-filter; this

Table 1. *Airborne bacteria inside and outside plastic ventilated isolators*

	Settle plate counts*		Andersen sampler counts (total per ft.^{3} of air)		
	Mean counts per plate	No. of observations	Expt. 1 (quiet ward)	Expt. 2 (busy ward)	Expt. 3 (quiet ward)
Isolator with filters	0·1 (range 0–0·4)	5	0·2	< 0·01	0·03
Isolator with coarse filter only	—	—	—	0·5	0·22
Isolator with no filter	1·0 (range 0–2·2)	10	1·2	—	0·13
Open ward	46·7 (range 11·0–82·8)	15	2·4	7·3	2·3

* Mean counts of colonies on 3½ in. (8·8 cm.) plates exposed for 6 hr. Each observation represents a sampling with a number of settle plates on one day.

showed that air pumped into the isolator with no filters lost a considerable proportion of its bacterial content, presumably through deposition in the duct conveying air from the fan unit to the canopy. The tests with an Andersen sampler showed a smaller difference between the airborne bacteria in the open ward and those in the isolator without filters than between settle-plate counts from the same areas; from which it could be inferred that most of the bacteria settling in the air-duct were carried on the larger particles – a conclusion supported by the size distribution of bacteria-carrying particles (Table 2). Most of the bacteria in the open ward during a busy period were carried on particles of 5·5 μm. and above, but in isolators with no filter or with a pre-filter only, the majority of airborne bacteria were carried on particles ranging from 1 to 2 μm. in diameter; these included some staphylococci. Very low counts (in Expt. 2 no detectable bacteria) were obtained in samples from the isolator with both coarse and fine filters.

Open-topped plastic isolator and air-curtain isolator

A Vickers plastic isolator with open top was used to assess protection of patients against personal (especially manual) contact transfer without control of airborne infection; in the trial, it was compared with a 'Sterair' patient isolator in which air curtains control the access of airborne bacteria without affecting the transfer of bacteria by contact. Before the clinical trial, sets of 5–10 settle plates were

Table 2. *Particle size distribution of bacteria inside and outside plastic ventilated isolator*

Estimated particle size	Expt. 1. (quiet ward) (viable counts per 30 ft.³ of air)				Expt. 2 (busy ward) (viable counts per 60 ft.³ of air)			
	Isolator with filters	Isolator with coarse filter only	Isolator with no filter	Open ward	Isolator with filters	Isolator with coarse filter only	Isolator with no filter	Open ward
9·2 μm. and above	1	—	1	15* (1 *Staph. aureus*)	0	0	—	145
5·5–9·2 μm.	2	—	3 (1 *Staph. aureus*)	15 (1 *Staph. aureus*)	0	1	—	109
3·3–5·5 μm.	0	—	5	19	0	0	—	79
2·0–3·3 μm.	2	—	6	11	0	7	—	63
1·0–2·0 μm.	0	—	19 (2 *Staph. aureus*)	11	0	22	—	42
Less than 1·0 μm.	1	—	1	0	0	0	—	0

* *Staph. aureus* were counted only in Expt. 1.

exposed for 6 hr. simultaneously on the unoccupied bed inside each isolator, on a table outside the isolator but close to it, and on a table at some distance from the isolator (at one end of the ward).

Results

The results are shown in Table 3. The mean settle plate counts inside the open-topped isolator were only slightly lower than those on settle plates exposed outside but next to the isolator, which was standing in a cubicle with door open to the ward but little traffic through it; though much higher counts were obtained on plates exposed in the open ward which was full of patients than in the unoccupied cubicle, the small difference between settle plate counts in the cubicle and in the isolator was taken to indicate a free circulation of airborne bacteria from the environment to the isolator; the slightly higher counts obtained outside the isolator were probably due to the settlement of heavier particles which would not reach the top of the canopy.

Table 3. *Settle plate counts inside and outside isolators*

Isolator	Mean settle plate counts (total)*		
	Inside isolator	Outside isolator (near bed)	Open ward (remote from bed)
Air curtains ('Sterair' unit)	9·2 (range 2·5–16·8)	29·0 (range 20·5–42·9)	90·6 (range 50·5–179·8)
Open-topped plastic isolator	16·7 (range 9·3–21·5)	23·6 (range 17·2–31·5)	98·3 (range 79·0–153·3)

* Five tests were made in each isolator, with five or six plates exposed for 6 hr. in each test.

The mean settle-plate counts inside the air curtain were about one tenth of the mean counts in the remote ward air; the ward air near the air curtain gave lower settle-plate counts than remote ward air, presumably because of the removal and recirculation of air from this zone through the filters of the 'Sterair' isolator.

CONTROLLED TRIALS OF ISOLATORS

Three trials were made on patients in the Burns Unit of this hospital, with the following treatment and control groups:

Trial 1. Treatment in (*a*) plastic ventilated isolator with coarse filter (pre-filter) and main filter; (*b*) plastic ventilated isolator with pre-filter only; and (*c*) the open ward (control group).

Trial 2. Treatment in (*a*) plastic ventilated isolator with pre-filter and main filter; (*b*) plastic ventilated isolator with neither main filter nor pre-filter; and (*c*) the open ward (control group).

Trial 3. Treatment in (*a*) plastic isolator with open top; (*b*) 'Sterair' isolator (air curtains); and (*c*) the open ward (control group). The purpose of this trial was mainly to assess the relative importance of airborne and direct contact contamination and the effect of barriers against each of these routes of contamination used separately.

Conduct of trials

In each trial patients with burns of between 4 and 30% of the body surface, if considered eligible on clinical examination, were allocated in rotation to treatment groups (*a*) and (*b*) and to the control group (*c*). Patients were kept in these groups for periods up to 3 weeks.

Local treatment of burns was by exposure method or (more usually) by application of a cream containing penicillin (1000 units per gram) covered with dressings of gauze, cotton-wool and crêpe bandage; penicillin cream was applied for protection against *Strep. pyogenes* only (Lowbury, 1960). Cloxacillin (250 mg. 6-hourly) was given by mouth to all patients in the first week, partly as prophylaxis against tetanus in those not known to be immune. Specific chemoprophylaxis against *Staph. aureus* and Gram-negative bacilli was not used; when such treatment was needed, patients were not put into the trial of isolators.

In Trial 3 a degree of barrier nursing was used for all patients in isolator and control groups; the precautions included individual washing bowls and bed pan supports, which were disinfected after use, and separate supplies of bed linen; they did not include the use of plastic or rubber gloves, apart from those incorporated in the plastic isolators. Barrier nursing was not used for the control groups in Trials 1 and 2.

Bacteriology

Swabs moistened with peptone water were taken from burns at every change of dressings, or daily if treatment was by exposure; the swabs were inoculated on horse blood agar (with 4% New Zealand agar), on 0·03% cetrimide agar and in cooked meat broth, which were incubated at 37° C. and examined in the manner described by Lowbury (1960) and Cason *et al.* (1966). Nasal swabs were taken daily and examined for coagulase-producing staphylococci (*Staph. aureus*). Antibiotic sensitivity tests were made by a ditch plate method (Topley, Lowbury & Hurst, 1951; Davis, Lilly & Lowbury, 1969) on all strains of *Staph. aureus* from burns and noses. Stool specimens or, if stools were unobtainable, rectal swabs were taken from all patients on admission and at intervals during the course of treatment; these were examined for Gram-negative bacilli by the methods used for burn swabs.

Results

Table 4 shows the comparability of patients in the treatment and control groups of the trials. The age of patients, areas of burn, and proportion treated by cover and by exposure methods fell within a similar range in each group.

Table 5 shows the incidence of infection of burns with *Staph. aureus* resistant to two or more antibiotics (multi-resistant or 'hospital' strains), *Ps. aeruginosa*, *Proteus* spp. and miscellaneous Gram-negative bacilli (coliform bacilli) in the trials of plastic ventilated isolators (Trials 1 and 2). Results entered as '+' refer to growth occurring on blood agar as well as in liquid medium; 'CM' refers to growth occurring only in liquid medium (cooked meat broth) and therefore very scanty.

Patients treated in isolators had a significantly lower incidence of infection with *Ps. aeruginosa* (4/37, 11%) than those in the control group (11/17, 65%) ($\chi^2 = 14{\cdot}5$, $P < 0{\cdot}001$); this applies to patients in isolators without filters as well as to those in isolators with filters. Though multi-resistant *Staph. aureus* appeared on burns more often in the open ward than in isolators, the difference was not significant. *Proteus* spp. and miscellaneous coliform bacilli appeared on burns at least as often in isolators as in the open ward.

Table 4. *Controlled trials of isolators: comparability of groups*

	Trials 1 and 2			Trial 3		
	Group *a*	Group *b*	Group *c*	Group *a*	Group *b*	Group *c*
Number of patients...	20	17	17	10	10	10
Number in age groups:						
<5	5	10	7	5	5	9
5–10	6	4	4	2	3	1
10–20	7	2	4	2	2	0
20–30	1	0	2	0	0	0
>30	1	1	0	1	0	0
Mean area of burn (%)	13·5	17	14	11	9	11
Range (%)	(4–30)	(7–19)	(5–30)	(6–20)	(5–13)	(8–20)
No. treated by covered method	15	12	15	6	5	8
No. treated by exposure method	4	2	1	2	5	1
No. treated by mixed covered and exposure methods	1	3	1	2	0	1

Table 6 shows the frequency of nasal acquisition in Trials 1 and 2 of multi-resistant *Staph. aureus*; such colonization occurred more often (during the first week significantly more often) in the control series than in the patients treated in isolators. Like the burns, the noses of patients treated in isolators often acquired hospital staphylococci, showing the limited effects of protection against airborne and personal contact transfer with very incomplete control of contact transfer by fomites or food.

Table 7 shows the colonization of burns by different groups of bacteria in the treatment and control groups of patients in Trial 3. The numbers of patients are small, but this trial, like Trials 1 and 2, showed a significantly lower incidence of *Ps. aeruginosa* in the burns of patients treated in the plastic isolator than in those treated in the open ward, though in this trial the isolator had an open top allowing circulation of air from the ward to the patient. By contrast, patients treated in the 'Sterair' isolator behind air curtains showed as high an incidence of *Ps. aeruginosa* infection of burns as those in the open ward. The other groups of bacteria appeared as often in the burns of patients treated in the open-topped plastic isolator and in the 'Sterair' isolator as in those treated in the open ward. Multi-resistant ('RR') *Staph. aureus* was less often acquired by patients in the control group of this trial than in those of Trials 1 and 2, possibly because of the use of some barrier nursing

Table 5. *Controlled trials of plastic isolators: bacterial infection of burns*

Patients in	*Staph. aureus* (multi-resistant)			*Ps. aeruginosa*			*Proteus* spp.			Coliform bacilli			Total patients
	+	CM	%+ or CM	+	CM	%+ or CM	+	CM	%+ or CM	+	CM	%+ or CM	
Isolators with filters	12	1	65	2	0	10	9	3	60	17	1	90	20
Isolators with coarse filters	4	1	55·5	1	1	22·2	3	1	44·4	7	1	88·8	9
Isolators with no filters	3	2	62·5	0	0	—	1	2	37·5	7	0	87·5	8
Isolators (all types)	19	4	62·2	3	1	10·8†	13	6	51·1	31	2	89·2	37
No isolators (control)	10	4	82·3	11	0	64·7†	3	2	29·3	16	0	94·1	17

† $\chi^2 = 14{\cdot}5$, $P < 0{\cdot}001$.
\+ = growth on solid medium. CM = growth only in fluid medium (cooked meat).

techniques in the control series of Trial 3. In contrast with the findings on ventilated isolators in Trials 1 and 2, there was no hint of any protective effect against *Staph. aureus* by treatment in the open-topped isolator or in the 'Sterair' isolator.

Table 6. *Controlled trial of plastic isolators: acquisition of* Staph. aureus (*Trials* 1 *and* 2)

Patients in		Multi-resistant *Staph. aureus* (+ and CM)				Total patients
		In burns		In nares		
		Patients	%	Patients	%	
Isolators	Whole period	23	62†	20	54†	37
	1st week	15	40†	9	24*	
Control series	Whole period	14	82†	14	82†	17
	1st week	11	64†	10	59*	

* $\chi^2 = 4{\cdot}4$, $P < 0{\cdot}05$. † Not significant.

PROBABLE SOURCE OF INFECTIONS

Cross infection and self-infection

Table 8 shows the incidence on admission of multi-resistant *Staph. aureus* in the nose and of Gram-negative bacilli in rectal swabs and stools of patients in the trials of isolators, in relation to the subsequent isolation of these organisms from the patients' burns. Out of 43 patients whose burns subsequently yielded *Staph. aureus*, only three had such an organism in the nose on admission. *Ps. aeruginosa* and *Proteus* spp. were usually absent from admission rectal swabs, though often acquired by burns later; other Gram-negative bacilli were usually present in rectal swabs on admission, but these did not include multi-resistant *Klebisiella* spp. which often appeared subsequently in burns. The results suggest that *Staph. aureus*, *Ps. aeruginosa*, *Proteus* spp. and *Klebsiella* spp. are usually acquired by cross-infection, while other Gram-negative bacilli (in particular *E. coli*) are acquired by self-infection from the patients' intestinal flora.

The predominance of cross-infection over self-infection with *Ps. aeruginosa* is also shown by the results of typing (see Table 9). Of the six patients from whom these data were obtained, two (Numbers 5 and 6) had *Ps. aeruginosa* in rectal swabs, one apparently acquired by cross-infection, but never had the organism in their burns. Another patient (Number 2) had *Ps. aeruginosa* in the burn but not in rectal swabs. One patient (Number 3) had two types of *Ps. aeruginosa*, both found in the Burns Unit; one never appeared in a rectal swab, the other appeared in a rectal swab after several previous negatives and after the same type had appeared in a burn. In one patient (Number 4) the rectal swab showed the strain of *Ps. aeruginosa* (of a type present in the Burns Unit) before it appeared in the burn, but there had previously been several negative rectal swabs. In patient Number 1 the strain (also of a type present in the Burns Unit) appeared at about

Table 7. *Controlled trial of isolators: air curtains and open-topped plastic isolator*

	Numbers of patients who acquired															
	In burns												In nares			
	Ps. aeruginosa			*Staph. aureus* RR			*Proteus* spp.			Coliform bacilli			*Staph. aureus*			
Isolator	+	CM	% + or CM	+	CM	% + or CM	+	CM	% + or CM	+	CM	% + or CM	+	CM	% + or CM	Number of patients
'Sterair' isolator (air curtains)	5	0	50*	4	0	40	3	3	60	7	3	100	3	2	50	10
Open-topped plastic isolator	0	0	0*†	6	1	70	4	0	40	8	0	80	3	0	30	10
Controls (open ward)	5	0	50†	3	1	40	2	1	30	6	1	70	3	0	30	10

* and †: $\chi_c = 2{\cdot}06$, $P < 0{\cdot}025$ (see Fisher & Yates, 1948).

the same time in a burn and in a rectal swab, after a negative rectal swab on admission. From these data it appeared that infection was usually acquired in hospital, though sometimes acquired first by the alimentary tract, from which it was transferred to the burns.

Contamination from fomites

Bacteriological samples were taken from a wide range of items supplied to patients in the ward; cotton-wool swabs moistened with peptone water were used for the sampling, and the bacteriological examination was made in the same way as that of swabs from burns.

Table 8. *Carriage of bacteria by patient on admission and subsequent infection of burns*

Bacteria	Site of carriage on admission	Bacteria carried on admission: Not in burns later	Bacteria carried on admission: In burns later	Bacteria not carried on admission but in burns later	Total: Sampled for carriage on admission	Total: Patients
Staph. aureus (RR)	Nose	0	3	40	73	84
Ps. aeruginosa	Rectum	2	1	18	54	
Proteus spp.	Rectum	0	4	23	54	
Coliform bacilli	Rectum	4	41	4	54	

From a number of items, of which 172 specimens were sampled (see Table 10), bacteria were grown, sometimes in moderate but usually in small or very small numbers. Patients in isolators (and also in the control group during Trial 3) had their washing bowls and disposable bedpan supports disinfected with 0·5% aqueous chlorhexidine solution. The bacteria usually found were multi-resistant *Staph. aureus* and miscellaneous coliform bacilli; *Ps. aeruginosa* and *Proteus* spp. appeared each in one specimen only. Of the 45 specimens of food, nine were contaminated with coliform bacilli. Even if personal contact and airborne transfer were completely excluded from patients in isolators, these fomites-borne contaminants might be expected to cause infection with staphylococci and with coliform bacilli in many patients.

THE NURSING OF PATIENTS IN PLASTIC ISOLATORS

Most of the patients treated in plastic isolators were children, and these usually accepted the isolation without complaint, sometimes with pleasure. The plastic canopy was virtually no barrier to conversation, and the patient did not feel cut off. Moreover, the visiting parent could touch the child through glove ports and did not have to wear cap, mask and gown. Adults were, on the whole, less happy about a prolonged stay in the isolator, and for larger patients the model of isolator with which we were supplied was too small for comfort.

The nursing care of patients in plastic isolators presented many problems. Such simple procedures as washing the patient or giving him a drink could be exhausting

Table 9. *Types of* Ps. aeruginosa *in faeces or rectal swab and on burns of patients in plastic isolators*

Patient	Date of admission	Isolator group	*Ps. aeruginosa* isolated: From burns — Serotype	From burns — Phage type	From burns — Date	From faeces or rectal swab — Serotype	From faeces or rectal swab — Phage type	From faeces or rectal swab — Date	Comments
1	14. iv. 68	Pre-filter and filter	3	16/31/68/F8/109/119X/352/M6/Col 11	21. iv.	—	Not typed	22. iv.	Burns Unit strain; rectal swab on admission negative (i.e. no *Ps. aeruginosa*)
2	10. xii. 68	Pre-filter and filter	—	Not typed (one isolate only)	20. xii.	—	None isolated	—	—
3	9. iv. 68	Pre-filter only	(*a*) 5*c* (*b*) NT	7/F7/119X 119X	14. iv. 27. iv.	5*c* —	7/31/F7/119X Not isolated	17. iv. —	(*a*) Recent Burns Unit strain (*b*) Current Burns Unit strain. First 4 rectal swabs negative
4	1. viii. 68	Pre-filter only	NT	119X	14. viii.	NT	119X	11. viii.	Burns Unit Strain. First 4 rectal swabs negative
5	19. v. 68	Pre-filter only	—	None isolated	—	NT	119X	27. v.	Burns Unit strain. First 5 rectal swabs negative
6	29. vii. 69	No filter or pre-filter	—	None isolated	—	3	21/31/44/68/F7/F8/109/119X	—	Not Burns Unit strain. First rectal swab negative

and frustrating to both nurse and patient. During the trials many improvements were made in the design of the isolator to facilitate nursing. Sleeves of glove ports were lengthened and made of more pliable material; the seams were strengthened, with the result that they did not often tear while in use; the canopy was enlarged (but further enlargement is needed); the zip fasteners were moved to more convenient positions. In spite of these improvements many difficulties remained, especially in the more complex nursing and clinical procedures, such as changing of dressings, passing of gastric tubes, setting up of infusions and taking of X-rays, especially in a wriggling and screaming child. The change of dressing required an extra 15–20 min. compared with the usual time. Bandages applied in isolators

Table 10. *Contamination of various items issued to patients*

Items	Number of samples	Number of samples contaminated with: *Staph. aureus** +	*Staph. aureus** CM	Gram-negative bacilli +	Gram-negative bacilli CM
Books, papers, etc.	20	—	—	2	1‡
Washing bowls	16	1	3	—	3
Crockery, glassware	55	3	—	1	3
Cutlery	8	—	2	—	—
Clean pillows	7	1	—	—	1
Disposable bedpan supports	8	6	—	2	1†
Urine bottles	2	1	—	—	1
Toys	8	1	—	—	—
Receiving bowls	3	—	1	—	1
Foods (various)	45	1	1	4	5
Total	172	14	7	9	16

* All strains were found resistant to two or more antibiotics except those from food, which were not tested.

† *Ps. aeruginosa.* ‡ *Proteus* sp.

have tended to be insecure, and dressings have, in consequence, sometimes fallen apart. To overcome these difficulties an isolator with an invaginated 'half-suit' has been produced, but although this gave the nurse much better access to all parts of the isolator, she could not stand upright while wearing the half suit in such a small isolator. It has been easier to manage patients in the open-topped isolators, but even these were cumbersome.

One of the special difficulties has been to prop the patient in a comfortable sitting position. Lifting and turning a heavy patient are very difficult and, for some nurses, impossible. Although the patient can be seen clearly through the transparent plastic of a new canopy, after a few days the plastic becomes clouded and the inspection of the patient becomes more difficult.

USE OF ISOLATORS AND AIRBORNE BACTERIA IN THE WARD

Effect of ventilation on airborne bacteria

The Sterair isolator recirculated a large volume of air (about 1200 ft.^{3} per min.) through filters. This led to a reduction of airborne bacteria in the immediate vicinity of the isolator (see Table 3), but the effect was localized, and the mean settle-plate counts in the ward during periods when the Sterair fan was working (93·6 per plate, mean of 30 plates) were little lower than those found during periods when the fan was switched off (110·0 per plate, mean of 20 plates).

Change of filters

Viable counts of bacteria in the air of the ward were not increased during the careful removal and replacement of pre-filters. Mean total counts were 3·9 per ft.^{3} before (6 min. sampling), 3·7 per ft.^{3} during (4 min. sampling) and 3·0 per ft.^{3} after (6 min. sampling) the change of pre-filter.

Ps. aeruginosa *and other bacteria in air*

From the evidence of the controlled trials it appeared that *Ps. aeruginosa* was transferred by contact but not by air. Air sampling with a slit sampler on cetrimide agar has shown very few colonies of *Ps. aeruginosa* in the air of the ward. At a time when a patient heavily infected with *Ps. aeruginosa* was in the ward, three samples of 414 ft.^{3} of air showed no colonies of *Ps. aeruginosa* on cetrimide agar; when the infected patient made vigorous movements, three colonies of *Ps. aeruginosa* were obtained in a sampling of 414 ft.^{3} taken next to her bed. In air samples on phenolphthalein diphosphate agar taken on the same occasion, total counts ranging from 3·0 to 33 per ft.^{3} and presumptive *Staph. aureus* counts ranging from 0·1 to 2·4 per ft.^{3} were obtained. In the dressing station during the change of dressings of the patient heavily infected with *Ps. aeruginosa*, small numbers of *Ps. aeruginosa* were grown from the air; the highest count (about 0·1 per ft.^{3}) was obtained during the removal of old dressings. Colonies of *Proteus* spp. were almost as infrequent in air samples as those of *Ps. aeruginosa*.

DISCUSSION

The studies reported here were made in order to assess the efficacy of certain types of isolator when used for protective isolation of patients with burns; the practicability of nursing patients in such isolators; and the relative importance of airborne and personal contact transfer of bacteria, as judged by the protective value of isolators which blocked either one or the other or both of these routes.

Preliminary bacteriological tests showed that airborne contamination was largely excluded in a plastic isolator with filters; when one or even both filters were removed, there was still an appreciable exclusion of airborne bacteria, especially of those carried on larger particles which settle quickly (and are therefore likely to contaminate the patient). Air curtains also excluded a considerable

proportion of the bacteria carried by the ambient air, and the filtration of air recirculated by the 'Sterair' unit also led to some reduction in the airborne bacteria in the immediate neighbourhood of this isolator. In an open-topped plastic isolator, however, there was very little exclusion of airborne bacteria.

Because of the necessity of avoiding topical chemoprophylaxis in assessing the value of isolators, the controlled trials were made on patients with burns of small or moderate extent in whom the clinical hazards of infection were negligible. There was a constant and significant protective effect against *Ps. aeruginosa* in plastic isolators, whether filters were present or not, and even when the top of the canopy was removed. There was also a small protective effect against endemic hospital staphylococci (especially against early nasal acquisition) in ventilated isolators, but no hint of such an effect in the open-topped isolator; nor was there any evidence of protection against *Ps. aeruginosa* or *Staph. aureus* by the 'Sterair' isolator, or against *Proteus* spp. and coliform bacilli by any of the isolators.

These failures are disappointing, and show that a degree of structural segregation greater than that provided by air conditioned cubicles (Cason *et al.* 1966) was still insufficient to achieve a useful protective result except against *Ps. aeruginosa*. This is not surprising, for a single momentary break in the protective barrier during the course of 2 or 3 weeks is likely to allow penetration by contaminants, which are abundant in a burns ward. The plastic ventilated isolator gives considerable protection against airborne and personal contact (especially manual) transfer, but none against contact transfer by fomites or food, and these may have been the vectors that caused much staphylococcal infection even in isolators with filters; sampling of a number of items supplied to patients showed that these bacteria were often present on them. Although air curtains did not appear to prevent infection of burns, they reduced the amount of contamination with airborne bacteria, including staphylococci. The effects of reduced exposure to airborne staphylococci inside air curtains would probably become apparent if contamination with the same bacteria by manual and fomites-borne contact were as effectively controlled by barriers against these routes of infection.

The comparison of air curtains with an open-topped plastic isolator supported the view that *Ps. aeruginosa* is usually transferred by personal (especially manual) contact, more rarely by fomites, and not by air. This is consistent with the frequent presence of *Ps. aeruginosa* on the hands of nurses working in the Burns Unit and other areas where infection with the organism is common and with the rarity of *Ps. aeruginosa* in air samples taken in the ward (Lowbury & Fox, 1954; Lowbury *et al.* 1970). The airborne transfer of *Ps. aeruginosa* in a dressing station for burns (Lowbury, 1954) must be regarded as exceptional, and due to the dispersal of *Ps. aeruginosa* surviving in dried exudate on removing dressings from extensive, heavily infected burns. Evidence from typing of *Ps. aeruginosa* and from rectal swabs supports the view that self-infection is rare, though sometimes infection of burns may be preceded by ingestion of the organism and its excretion in the faeces. With the other types of bacteria, since there was little or no difference in the acquisition of these by patients in the two types of isolator and in the controls, it seems that neither airborne nor direct contact transfer plays the predominant role. Since

the combined protection against airborne and direct contact transfer reduced the amount of staphylococcal infection when protection against neither route by itself had this effect, it could be inferred that staphylococci were transferred both by air and by direct contact. Since much infection occurs when both routes are blocked, indirect contact contamination by fomites, food, etc., also seems important in the transfer of staphylococci.

The patients for whom isolators, if effective, might be considered potentially valuable are those with extensive burns. The protective value of isolators for such patients is likely to be smaller (certainly not greater) than it has been shown to be for the less extensive burns studied in our trials; the difficulty of nursing burned patients in isolators, however, is even greater when the burns are extensive than when they are of small or moderate severity. In view of the success of local chemoprophylaxis by silver compounds and other agents in keeping burns free from many types of bacteria, it seems unlikely that isolators will play a large role in the routine treatment of burns in hospital. But the significant protection by plastic isolators against *Ps. aeruginosa* gives this method a role in the treatment of certain patients, e.g. those in whom effective topical agents cause toxic or allergic effects. If their use is restricted to this extent, it might be practicable, from the nursing angle, to use the full range of precautions against contamination by food and fomites as well as against manual and other personal contact contaminations. But though improved design of isolators should facilitate their use in selected patients, experts trained in their use will be needed. Since burns often become infected with one pathogen while remaining free from others, it is important that isolators used in the treatment of burns should be suitable for containment of bacteria (by the use of filters in the air-effluent) as well as for protection against contaminants.

Unlike the plastic isolator, air curtain isolators present no difficulties in the nursing or medical treatment of patients. Unfortunately, they also show no sign of giving the patient any useful protection against contaminants – at least, when used without other effective barriers. It is possible that air curtains might be found to have some value if nurses and others wore gloves and protective clothing when attending to patients inside them, but this hypothesis cannot be accepted without further study. It seems likely too, that a physical barrier, such as that provided by the open-topped isolator, is valuable not only because it gives protection against contamination from the hands and uniform of nurses, but because it acts as a barrier against accidental contamination and social contacts with visitors who are not familiar with the rules of hospital hygiene.

In a parallel study (Ayliffe, Collins, Lowbury & Wall, 1971) it was found that patients in a self-contained, plenum-ventilated isolation suite with air-locks were protected against nasal acquisition of *Staph. aureus*. A plastic ventilated isolator might be expected to give a higher degree of protection than isolation in a hospital room, but the burns ward where isolators were used presented a much greater challenge of contamination than that to which the isolation suite in a clean surgical ward was exposed. The high degree of isolation provided by the suite with air locks must also have contributed to the good result with this form of isolation.

We wish to thank the Department of Health and Social Security for their support of this work, Dr O. M. Lidwell and Dr P. C. Trexler for valuable suggestions, Messrs Vickers Limited for the supply of isolators during our pilot studies, W.H.S. Pathfinder Ltd for the use of Fig. 4, Dr M. T. Parker for the typing of strains, Mr W. H. Cater for technical assistance, Dr J. P. Bull for statistical advice, and the nursing, clinical and laboratory staff for their co-operation.

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EXPLANATION OF PLATES

Plate 1

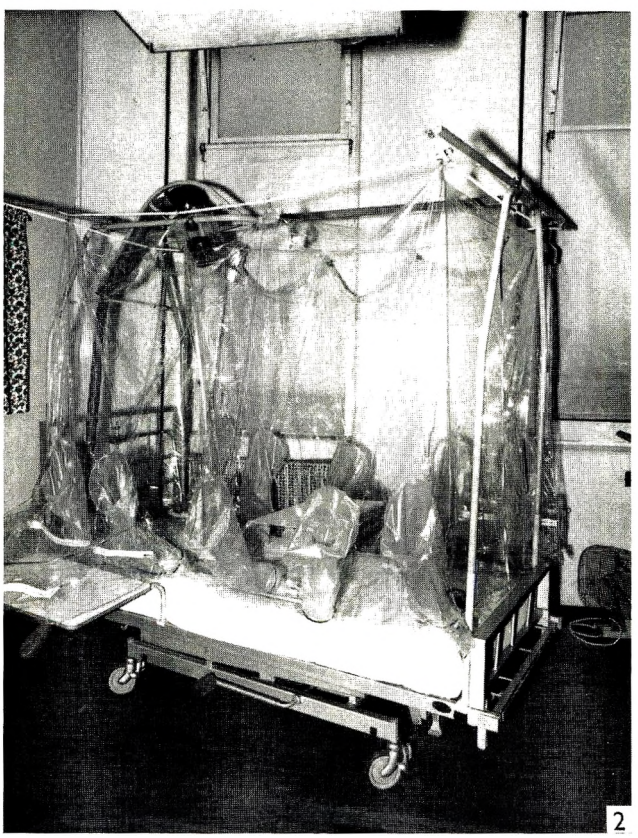
Fig. 1. Plastic ventilated isolator. The isolator, in which a patient is having dressings changed, is equipped with a half-suit and ventilated headpiece to facilitate nursing.

Fig. 2. Plastic isolator with open top.

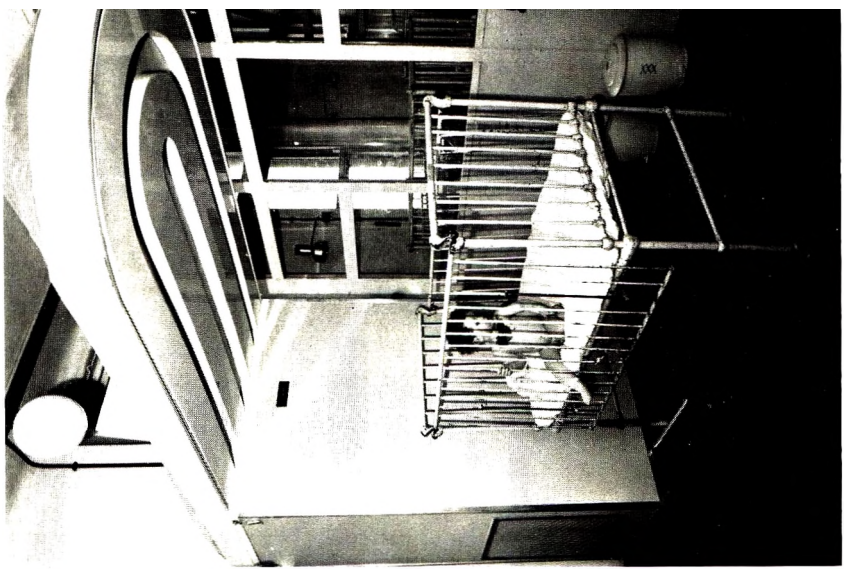
Plate 2

Figs. 3 and 4. Air curtain isolator. Air is drawn through grids on each side of the console at the head of the bed, filtered, and pumped out through slits on the under surface of the canopy over the bed. The air curtain is illustrated in the diagram (Fig. 4).

1



2



4

Diagrammatic representation of air curtain effect

3

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Trials of intranasally administered rubella vaccine

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SUMMARY

No evidence of vaccine virus transmission was found in two studies where Wistar RA 27/3 rubella vaccine was administered intranasally. Vaccine was immunogenic in all of 23 vaccinated children in one study, while in the other only 5 of the 11 vaccinees developed antibody. The reduced seroconversion rate in the latter study appears to have been caused by one or a combination of factors, including the vaccination technique, the presence of infective nasal conditions in vaccinees and the titre of vaccine used.

INTRODUCTION

The intranasal method of administering a vaccine is as convenient and at least as acceptable to recipients as the subcutaneous injection method. Furthermore, intranasal vaccination may produce local immunity at the portal of entry of natural infection, as well as stimulating circulating antibodies, as suggested by Smith (1969). However, in theory, the propensity of a vaccine effective intranasally to spread to susceptible contacts might be greater, and it is conceivable that an intranasally administered vaccine could be less effective if there was a pre-existing local bacterial infection or an allergic condition. The Wistar RA 27/3 attenuated rubella vaccine has now been licensed for subcutaneous administration in the United Kingdom and Republic of Ireland, although not as of now in the United States. It is, however, the only vaccine which is immunogenic when administered intranasally. Because of the theoretical risk of administering extraneous agents with vaccines the intranasal route may be a safer method of using the vaccine. Although preliminary studies by Ingalls, Plotkin, Philbrook & Thompson (1970) suggest that transmission of virus to susceptible contacts following intranasal vaccination is unlikely to constitute a problem, there is probably insufficient published data to justify recommending intranasal inoculation in the general community.

This paper reports on clinical experiences with Wistar RA 27/3 strain rubella vaccine in the first extended trials of intranasally administered vaccine in Ireland and Britain. Two studies were carried out. The first was in a group of children residing for social reasons in an orphanage in the suburbs of Dublin. The second was conducted in a manner similar to that of an earlier investigation of subcutaneously administered Wistar RA 27/3 rubella vaccine (Hillary *et al.* 1969). This second intranasal study took place in a semi-rural area covering 200 square

miles (510 km.^{2}) in the Irish midlands during the 1970 school summer holidays. The area was selected because a previous survey for rubella antibody showed a relatively high proportion of seronegatives in school-children (Hillary, 1971). In all studies, stringent precautions were taken to avoid any possible infection of a pregnant woman from a vaccinated child. Contacts were observed for both clinical and serological evidence of vaccine virus spread.

MATERIALS AND METHODS

Study populations

Study 1

Sixty-nine children aged 2–18 years resident in the orphanage were bled and rubella haemagglutination inhibiting (HAI) antibody titrations were carried out. Seventeen children were found to be rubella seronegative with HAI antibody titres of $< 1/10$. Eleven of these and one seropositive child were vaccinated. The remaining 6 seronegative children in frequent contact with the vaccinated children were retained as indicators of vaccine virus transmission. All children involved in this study showed clinical evidence of upper respiratory infection at the time of vaccination. Nose and throat swabs were taken from vaccinees and contacts on the 8th, 10th and 12th days after vaccination. Vaccinated children were examined clinically on alternate days up to the 28th day after vaccination. Contacts were seen twice during the 14th- to 21st-day period. Blood samples were collected from both vaccinees and contacts 9 weeks after vaccination.

Study 2

Sixteen mothers of large families in a semi-rural area in the Irish midlands gave permission for their children to take part in this study after an explanation of its purposes and procedure. Of 72 children aged between 2 and 15 years, 53 were seronegative (HAI titre $< 1/10$). Of these, 23 females were vaccinated. The remaining 30 (11 females and 19 males) were retained to determine whether virus transmission occurred. Each vaccinated child had at least one non-immune sibling living in the same house (Table 1). There was no clinical evidence of upper respiratory tract infection in any of the participants in this study. Vaccinees only were clinically examined on the 8th, 10th and 12th days after vaccination, while contacts and vaccinees were examined on the 24th and 26th days. Blood samples were collected from both vaccinees and contacts 8 weeks after vaccination.

Serology

In the first study, rubella HAI antibody titrations were carried out using the technique described by Stewart *et al.* (1967), pigeon red cells being substituted for chicken cells as the indicator material on grounds of convenience (Peetermans & Huygelen, 1967). In the second study manganous chloride and heparin were substituted for kaolin for the removal of non-specific inhibitors (Mann, Rossen, Lehrich & Kasel, 1967; Plotkin, Bechtel & Sedwick, 1968), but otherwise the technique was unchanged.

Table 1. *Age and immune status of vaccinees and siblings*

	Age in Years														
Family	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	.	.	0	.	.	0	−	+	.	+	+	.	.	.	.
B	.	.	.	.	0	.	.	−	0	.	.	−	−	.	.
C	.	.	.	.	0	.	−	.	+	.	+	.	+	.	+
D	.	.	.	.	.	.	.	.	0	−	.	.	.	.	.
E	.	.	.	.	.	.	−	−	.	.	0/0	.	.	.	.
F	.	.	.	.	.	.	.	.	−	.	.	.	0	.	−
G	.	.	.	.	0	0	−	.	−	.	.	.	.	.	.
H	.	.	−	0	.	.	.	.	.	.	.	.	.	.	.
I	.	.	.	0	.	.	.	−	.	+	.	+	.	+	.
J	.	.	.	.	0	.	−	.	.	.	0	+	+	−	.
K	.	.	.	−	−	.	.	.	−	+	.	.	0	.	.
L	.	.	.	.	.	.	.	.	.	.	.	−	.	0	+
M	.	−	0	.	−	.	.	−	0	−	.	.	.	.	.
N	.	.	.	.	.	.	.	.	.	.	0	−	.	.	.
O	.	−	−	.	.	0	0	.	.	.	+	+	.	+	.
P	.	.	−	.	−	.	−	.	+	0	+	.	.	.	.

0, Non-immune vaccinees (total 23); −, negative contacts (total 30); +, positive contacts (total 19).

Vaccine

In study 1, 27th passage level Wistar RA 27/3 titre of $10^{2\cdot94}$ TCID 50/dose was used.

In study 2, 28th passage level Wistar RA 27/3 titre of $10^{4\cdot1}$ TCID 50/dose was used.

In both studies freeze-dried vaccine was reconstituted in 0·5 ml. of diluent. For vaccination in the first study children were seated in low chairs with their heads hyperextended and 0·25 ml. reconstituted vaccine was administered as nose drops in each nostril. In the second study children lay flat on couches with their unsupported heads hyperextended. This position was maintained for at least 1 min. after the administration of vaccine.

RESULTS

Study 1

Only 5 of the 11 susceptible vaccinees developed antibody following administration of vaccine and only in 4 of these was a clear fourfold or greater increase in titre seen. Nine-week post-vaccination titres of these subjects were 1/20, 1/80 (2), 1/60 (2). None of the 6 contacts developed HAI antibody.

Nasal and throat swabs cultured bacteriologically showed *Staphylococcus aureus*, *Staphylococcus albus*, *Streptococcus viridans*, pneumococci, diptheroids and commensal neisserias. Little difference was evident in the organisms cultured from those responding and those not responding to vaccine administration. Reactions seen following the administration of vaccine are recorded in Table 2.

Study 2

All vaccinated children showed a four-fold or greater rise in antibody titre (Table 3). None of the sibling contacts developed antibody.

Reactions seen in vaccinated subjects were few and included enlarged cervical lymph nodes, rash, tonsillitis and upper respiratory infection with cough. These reactions are summarized in Table 4. Enlarged occipital lymph nodes were also seen in control subjects, but were attributed to *Pediculus capitis* infestation, since no change in serological status was evident in any contact child.

Table 2. *Reactions seen following administration of vaccine – study* 1

Nature of reaction	No. of children	Mean day of onset	Duration (days)
Palpable post-auricular glands	2	9	$3\frac{1}{2}$
Cough	1	9	2

Table 3. *Titres* 9 *weeks after vaccination – study* 2

	Rubella HAI antibody titrations (reciprocals)							
	Before vaccination	After vaccination						
No. of children	< 10	< 10	= 10	20	40	80	160	320
23	23	0	0	0	2	6	11	4

Post-vaccination modal titre: 160; median titre: 160; G.M.T.: 133.6.

Table 4. *Reactions seen following administration of vaccine - study* 2

	No. of children		Mean day of onset		Duration (days)	
Nature of reaction	Vaccinees	Controls	Vaccinees	Controls	Vaccinees	Controls
Lymphadenopathy	16	4	10	0	7	14
Rash	2	0	10	0	1	0
Tonsillitis	2	0	12	0	2	0
Pyrexia > 100° F.	1	0	10	0	2	0
Cough with upper respiratory infection	2	0	12	0	2	0

DISCUSSION

In neither of these studies was there evidence of spread of vaccine virus from vaccinees to susceptible contacts. The conditions in favour of transmission of infection are maximal between children in large-family groups with close and frequent personal contact, particularly where there is a high incidence of rubella susceptibility. Children living in orphanages are less sensitive indicators of vaccine virus spread, since there is less direct contact between the vaccinee and susceptible unvaccinated children. Vaccine at a titre per dose of 10,000 TCID50 was immunogenic in all vaccinated children. The low seroconversion rate seen in the children in the orphanage study, where only 5 of 11 developed antibody, may have been caused by one or a combination of several factors – the vaccination

technique, the presence of infective nasal conditions at the time of vaccination and the lower titre of vaccine used.

Maximum contact between nasal and nasopharyngeal mucosa and vaccine is more likely to be obtained when subjects lie flat with their heads fully hyperextended and where this position is maintained for a brief period following intranasal administration of vaccine. When children are seated, although their heads are hyperextended, vaccine tends to flow over the nasal surfaces of hard and soft palates and be swallowed, allowing a relatively shorter time in contact with the nasal mucosa.

Vaccine at a titre of 500 TCID 50 has been shown to be fully immunogenic when given intranasally (Plotkin, Farquhar, Katz & Buser, 1969). Thus the titre of vaccine used in the orphanage study may be significant only in the context of the vaccination technique used and the condition of the noses. The presence of nasal virus or bacterial infection may directly interfere with the ability of the vaccine to infect susceptible cases and in addition nasal discharge or nasal obstruction may physically limit contact between vaccine and nasal mucosa. Further work is needed to clarify the relative importance of these factors and also to determine whether vaccination by the intranasal route has advantages in addition to those of acceptability and convenience. The presence of local immunity is acknowledged to be of considerable importance in protection against other virus infections (Tyrrell, 1969).

If intranasal administration of Wistar RA 27/3 produces local nasal IgA antibody this may limit the re-infection of vaccinated subjects exposed to natural infection (Horstmann *et al.* 1970; Chang, Desrosiers & Weinstein, 1970). Thus investigations are required to determine the extent to which local immunity is produced by rubella vaccines and the degree to which the presence of nasal antibody protects against natural or artificial challenge.

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An M-associated protein antigen (MAP) of group A streptococci

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SUMMARY

A streptococcal antigen that is closely associated with the M-antigen, but is not type specific can be detected by means of a complement-fixation test in extracts of M-positive, but not of M-negative, variants of group A streptococci. Purification of acid extracts results in a concomitant increase in the purity both of the type-specific M-antigen and of the M-associated protein (MAP). Antibody to MAP is present in the sera of patients who have had streptococcal infection. The highest titres are found in patients with rheumatic fever.

INTRODUCTION

Group A streptococci that synthesize a type-specific M-antigen will multiply in normal human blood. If the corresponding antibody is added to the blood the streptococci are opsonized and killed by phagocytosis (Todd, 1927). The addition of extracted M-protein of the homologous type will neutralize the antibody and prevent this bactericidal action. During investigations into the M-protein content of fractions obtained from group A streptococci, we observed that, in the blood of some donors, the bactericidal action on M-positive streptococci in the presence of homologous rabbit antiserum was also neutralized by extracts of streptococci of heterologous M-types.

MATERIALS AND METHODS

Strains

Group A streptococci were stock laboratory cultures and cultures that had been submitted to the Streptococcus Reference Laboratory for routine typing. Members of other groups were stock cultures. Paired M-positive and M-negative variants of various types were also available (see Widdowson, Maxted & Grant, 1970).

Medium

The growth medium was Oxoid Todd-Hewitt Broth (Oxoid Ltd., London) with the addition of 1% (w/v) Neopeptone (Difco Ltd., Detroit, Michigan, U.S.A.).

Rabbit sera

M-antisera against whole heat-killed streptococcal cells were prepared in the Streptococcus Reference Laboratory.

Human sera

Sera from cases of rheumatic fever, glomerulonephritis and uncomplicated streptococcal infection were obtained from hospitals and Public Health Laboratories. 'Normal' sera had been submitted for Wassermann tests or were from laboratory workers.

The bactericidal test

This was carried out as described by Maxted & Valkenburg (1969). In tests in which streptococcal extracts were added to the bactericidal system 0·02 ml. quantities of extract were added to 0·02 ml. of M-antisera prepared in rabbits; 0·02 ml. of a suitable dilution of a 3–4 hr. culture of streptococci of a homologous type (containing 50–200 colony-forming units per 0·02 ml.) was added to the mixture in small glass tubes; 0·3 ml. of heparinized human blood was added to each tube, and the tubes were sealed and incubated at 37° C. for 3 hr. in a rotating drum. The tubes were opened, and 0·02 ml. quantities of the reaction mixture were inoculated into pour plates of Hartley digest agar (Cruickshank, 1965) together with 10% (v/v) horse blood. After 24 hr. at 37° C. the plates were examined and survival was scored as follows: + + + + = confluent growth, + + + = > 200 colonies, + + = 50–200 colonies, + = up to 50 colonies and − = no colonies.

The complement-fixation test (CFT)

This was performed as described by Bradstreet & Taylor (1962). Various streptococcal fractions were used as antigen, and the complement dose was three times the minimum haemolytic dose (3 MHD). Fixation was allowed to take place overnight at 4° C. After the addition of the sensitized sheep red cells the tests were incubated at 37° C. for 30 min. The results were recorded after 2 hr. at 4° C. as + + + + = no haemolysis, + + + = slight haemolysis, + + = 50% haemolysis, + = about 75% haemolysis, 0 = complete haemolysis.

Many human sera tested were anti-complementary in preliminary tests. This was eliminated by incubation of the serum with 3 MHD of complement at 37° C. for 30 min. The sera were then inactivated at 56° C. for 30 min.

Preparation of streptococcal extracts

Hot-acid extracts were made with 0·2 N HCl by the method of Lancefield (1928) and formamide extracts by the method of Fuller (1938). Purified M-antigens were prepared from crude 0·2 N acid extracts by precipitation at pH 2, ribonuclease treatment and ammonium sulphate fractionation (Lancefield & Perlmann, 1952), carboxymethyl cellulose chromatography (Fox & Wittner, 1965) and polyacrylamide gel electrophoresis (Widdowson, Maxted, Grant & Pinney, 1971). M-antigens of various types were purified, and at each stage of purification the

volume of the fraction was recorded and the protein content per ml. was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The complement-fixation titre at each stage was determined by titration of twofold dilutions of the fraction with suitable dilutions of the test human serum. The titre of type-specific M-protein of each fraction was determined by a gel-diffusion test of twofold dilutions of the fraction against appropriate absorbed specific M-antiserum.

Absorption of human sera

All absorptions were carried out at 4° C. for 18 hr. For absorption with whole streptococcal cells, overnight broth cultures were centrifuged and the cells washed three times in 0·85% (w/v) saline. The packed cells (1 cm. depth in $3 \times \frac{1}{2}$ in. test tubes) were used to absorb 1 ml. of the serum diluted 1/10 in phosphate-buffered saline pH 7·5. Membranes of a type 49 M-positive strain were prepared by the phage–lysin method of Freimer (1966). Washed membranes were suspended in saline and the optical density was adjusted to 1·5 at 600 nm. in a Unicam SP 600 Spectrophotometer. This suspension was mixed with an equal volume (1 ml.) of 1/10 dilutions of the sera under test. Samples of normal human heart and skeletal muscle and rabbit heart, kidney and skeletal muscle were finely minced and washed in normal saline until the washings were clear. The minced tissue was strained through muslin and used (1 cm. depth of packed tissue in $3 \times \frac{1}{2}$ in. test tubes) to absorb 1 ml. quantities of 1/10 dilutions of serum.

Anti-streptolysin O tests

These were performed by the method of Gooder & Williams (1959).

Streptococcal anti-DNAse B tests

These were done by the method of Nelson, Ayoub & Wannamaker (1968).

RESULTS

Addition of extracted M-antigen to the bactericidal system

The effect of adding crude acid extracts of group A streptococci to the indirect bactericidal system (M-positive streptococcus + homologous rabbit antiserum + fresh human blood) is summarized in Table 1. The expected result was obtained with the blood of 8 out of 10 normal donors; only the addition of extracted M-antigen of the homologous type resulted in the survival of the streptococcus. However, the blood of two donors, who had no history of recent streptococcal infection, gave a different result; the addition of acid extracts of M-positive, but not of M-negative, streptococci of any type abolished the bactericidal effect. Table 1 also shows the results of addition of acid extracts of M-positive and M-negative variants to the direct bactericidal system, where the survival of an M-negative streptococcus, usually unable to multiply in human blood, was promoted in the presence of M-positive extracts, but again only in the blood of the same two abnormal donors. Formamide extracts, which contain the group carbohydrate but no M-antigen, had no effect on either system.

Although there is evidence that complement is involved in the phagocytosis of M-negative streptococci, the participation of complement in the phagocytosis of M-positive cells opsonized by type-specific antibody has been questioned (Stollerman, Rytel & Ortiz, 1963; Stollerman, Alberti & Plemmons, 1967; Saito, 1970). However, the requirement for small amounts of complement, in addition to the heat-labile opsonins described by Stollerman *et al.* (1963), cannot be ruled out, and we considered that the observed reversal of the bactericidal effect in both the direct and indirect systems might be explained on the basis of complement depletion by an extraneous antigen–antibody system, and that the antigen involved was associated with M-antigen.

Table 1. *The effect of addition of extracted M-antigen in the bactericidal system*

Present in all tubes	Additions		Amount of growth after 3 hr. at 37° C.*	
	Rabbit antiserum	Extract	Expected result	Result with blood of certain donors
Human blood + M-positive group A streptococcus	Nil	Nil	+ + + +	+ + + +
	Homologous anti-M serum	Nil	−	−
	Homologous anti-M serum	Acid extract of homologous M type	+ + + +	+ + + +
	Homologous anti-M serum	Acid extract of any heterologous M type	−	+ + + +
	Homologous anti-M serum	Acid extracts of M-negative variants	−	−
	Homologous anti-M serum	Formamide extract of M-positive streptococcus	−	−
Human blood + M-negative group A streptococcus	Nil	Nil	−	−
	Nil	Acid extract of any M-positive streptococcus	−	+ + + +
	Nil	Acid extracts of M-negative variants	−	−
	Nil	Formamide extract of M-positive streptococcus	−	−

* 0·02 ml. of reaction mixture was inoculated into pour plates of Hartley digest agar + 5 % (v/v) horse blood; + + + + = confluent growth; − = no growth (see text).

Detection of M-associated antigen by complement-fixation tests with sera of abnormal donors

To test the hypothesis that the sera of the abnormal donors contained an antibody that fixed complement in the presence of streptococcal extracts, we performed complement-fixation tests on these sera with 0·2 N acid extracts of M-positive streptococci of 50 different types, and with M-negative variants obtained from streptococci of 10 different types. The sera of both the abnormal donors fixed complement (one at a dilution of 1/40 and the other at 1/20) in the presence of a 1/50 dilution of all the Lancefield extracts of M-positive streptococci, but not in the presence of

the extracts of M-negative streptococci. No fixation was obtained with the sera of 'normal' donors at a dilution of 1/4 even when tested with undiluted extracts of the streptococci. Titration of acid extracts of M-positive strains in complement-fixation tests with a fixed dilution of 'abnormal' serum gave titres of at least 80, and often as high as 320 or 640. In general, a high CFT titre was associated with a high titre for type-specific M-antigen in the extract. Fixation by M-negative extracts was not observed at dilutions higher than 1/5. Formamide extracts of M-positive variants did not fix complement in the presence of the serum of abnormal donors.

Lancefield extracts of representative strains of streptococci of groups B to R were tested for complement fixation with a suitable dilution of the serum of an abnormal donor. In general, complement was not fixed with extracts of streptococci of groups other than A. The exceptions were certain group C and G strains that were capable of survival in human blood, and thus might be assumed to have M-like antigens. Extracts of group G strains known to have type 12 M-antigen (Maxted & Potter, 1967) also contained the complement-fixing antigen.

Sensitivity of the M-associated antigen to proteolytic enzymes

The ability of extracts of M-positive strains to precipitate with their type-specific antiserum, to fix complement with the sera of abnormal donors, or to produce an apparent non-specific neutralization effect in the bactericidal test with the blood of these donors, was abolished by treatment at 37° C. with trypsin, pH 7·8, in 15 min., with pepsin, pH 5·2, in 15 min., and with streptococcal proteinase (Elliott, 1945) in 2 hr.

Purification of M-associated protein antigen (MAP)

Crude acid extracts of strains of M-type 5 and M-type 30 were prepared and purified by precipitation at pH 2·0, ribonuclease treatment, ammonium sulphate fractionation and carboxymethyl cellulose chromatography.

The protein content per ml., the CFT titre and the titre of type-specific M-protein were determined at each stage. Table 2 shows the results obtained with the type 5 strain. The minimum quantity of protein required to give a positive reaction in tests for both antigens was determined at each stage by dividing the protein content of the extract by the titre of the reactions, and this was used as a measure of the purification of the antigens. About 10 times more protein was required to give a positive precipitin line by gel diffusion with the homologous M-antiserum than to give a positive CFT with the serum of an abnormal donor. The purity of the two antigens was increased in almost identical ratio (1 to 7·3–7·4) by precipitation with 60% saturated ammonium sulphate, and by subsequent fractionation of this precipitate on carboxymethyl cellulose (1 to 26·7). The results of fractionation of the P 60 fraction (i.e. the precipitate obtained between 33% and 60% saturation with ammonium sulphate) on carboxymethyl cellulose with a pH gradient from 4·0 to 7·0 showed that the material in the second peak (pH 7·0) had higher activities for both proteins than the first peak (pH 5·5) which contains proteins other than M-protein and probably residual nucleic acid (Fox & Wittner, 1965).

Table 2. *Purification of M-antigen and MAP from a crude Lancefield extract of a type 5 strain*

Fraction	Volume (ml.)	Protein (mg./ml.)	*M-antigen titre	†CFT titre	Minimum quantity (μg./ml.) of protein required for a positive precipitin test‡	Minimum quantity (μg./ml.) of protein required for a positive CFT‡	Purification factor for M-antigen and MAP§
Crude extract	260	2·55	8	64	320	40	1·0
pH 2 precipitate	22	6·10	32	256	190	24	1·7
pH 2 supernatant	233	2·1	0	0	—	—	—
33 % $(NH_4)_2SO_4$ precipitate	14	0·425	0	8	—	53	< 1
60 % $(NH_4)_2SO_4$ supernatant	39	0·575	1	16	575	36	< 1
60 % $(NH_4)_2SO_4$ precipitate	25	2·775	64	512	43	5·5	7·3–7·4
CMC 1st peak (pH 5·5)	—	0·630	8	64	76·5	10	4·0–4·2
CMC 2nd peak (pH 7·0)	—	0·390	32	256	12	1·5	26·7

* Highest twofold dilution to give a precipitin line in gel diffusion test with absorbed anti-M serum.
† Highest two-fold dilution to give a 2+ fixation in the CFT with a fixed dilution of an anti-MAP serum.
‡ Protein content/titre.
§ Calculated relative to crude extract.

Purification of the P 60 fraction of an M-type 30 strain by CMC chromatography showed that, as in the case of the type 5 strain, the second peak had higher specific activity than the first peak for both type-specific M-protein and MAP. Minimum quantities of protein to give a positive CFT for type 30 M-protein were: 5 μg./ml. of P 60 fraction, 5–10 μg./ml. – first peak, and 1–1·5 μg./ml.–second peak.

M-proteins of three other types (12, 24 and 49) were purified as far as the P 60 stage. Complement-fixation tests on these P 60 fractions showed that, as with the corresponding fractions of type 5 and type 30 M-protein, approximately 5 μg./ml. of protein was required to fix complement in each case.

Polyacrylamide gel electrophoresis of a P 60 fraction of a type 24 M-protein showed one main band and several minor ones. Both type-specific M-protein and MAP could be detected most strongly in the main band. The minor bands also contained both antigens.

The presence of anti-MAP in human sera

Twofold dilutions of sera from patients with various streptococcal disease were tested for complement fixation with a suitable dilution (determined by preliminary titration) of the purified P 60 fraction of M-protein from a type 30 strain. This type is rare in the U.K. and was chosen to avoid possible complement fixation by a type-specific antigen–antibody reaction. In some experiments, crude acid extracts

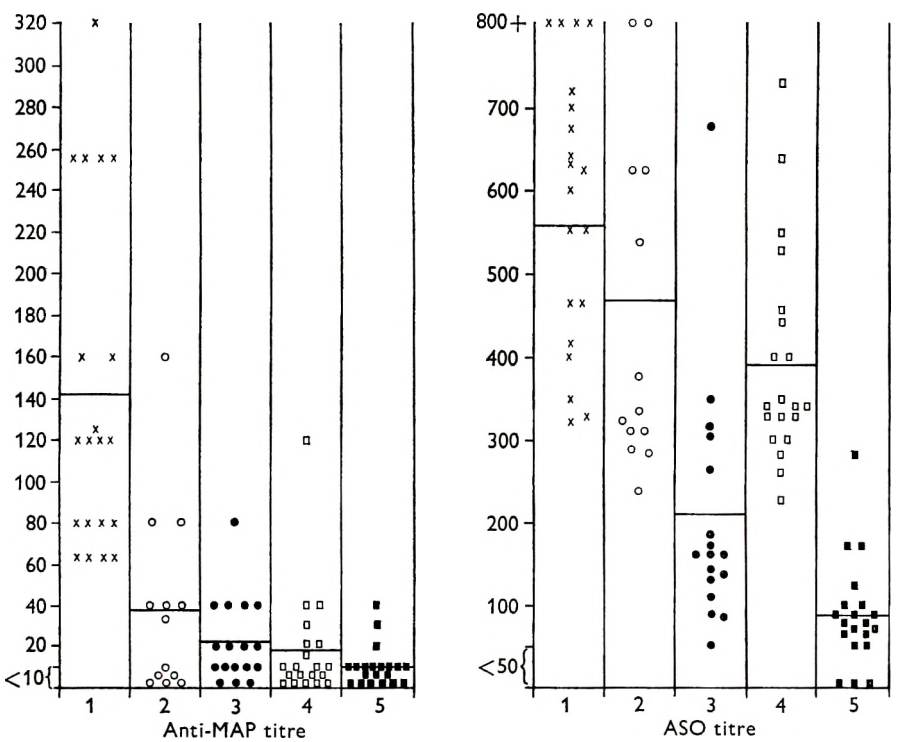

Fig. 1. Titres of anti-MAP and ASO in streptococcal diseases × (1) Rheumatic fever. ○ (2) Nephritis following throat infection. ● (3) Nephritis following skin infection. □ (4) Uncomplicated streptococcal infection (throat). ■ (5) Normals.

of various types were also used as antigen. Such extracts contain many impurities, and fixation due to antibodies to these seemed a possibility. However, in tests with human sera and crude acid extracts of M-positive and M-negative strains, and with formamide extracts, fixation occurred only with the extracts of M-positive strains, and the titre of the reaction was identical with that obtained with purified M-proteins whatever the M-type of the strain used. This suggests that the purity of the M-antigen may not be an important criterion in complement-fixation tests with human sera.

Fig. 1 shows the scatter of anti-MAP titres in various human sera. The arithmetical mean for each category is also shown.

The titre exceeded 60 in all of the 20 cases of rheumatic fever tested but only in 4 of 30 cases of acute nephritis and 1 of 30 cases of uncomplicated streptococcal infection. The average titre in rheumatic fever was 140, about 4 times higher than that of the other categories of infection considered together. The anti-MAP titre in 'normal' individuals was generally not greater than 10.

Although many of the patients with rheumatic fever had high ASO titres some did not, and the average titre in rheumatic fever was not greatly different from that seen in nephritis following throat infection and in uncomplicated streptococcal disease. In nephritis secondary to streptococcal skin sepsis, on the other hand, the ASO titre was seldom significantly raised (Potter *et al.* 1968; Anthony, Perlman & Wannamaker, 1967; Dillon & Reeves, 1969).

These results are summarized in Table 3, which also gives the anti-DNAse B titres for the cases of nephritis following skin infection. These confirmed that the patients had indeed suffered from a recent streptococcal infection (Kaplan *et al.* 1970). There is little doubt, therefore, that the anti-MAP response in nephritis is normally lower than that in rheumatic fever.

Removal of antibody to M-associated protein by absorption

The relative titres of anti-MAP in sera from patients with rheumatic fever and with the other streptococcal diseases are similar to the relative titres of heart-reactive antibody in the sera of comparable groups of patients (Zabriskie, Hsu & Seegal, 1970). The presence of heart-reactive antibodies in human and rabbit sera can be demonstrated by fluorescent staining of human or rabbit heart sections, and Zabriskie & Freimer (1966) and Kaplan (1966) have shown that the antibodies are absorbed by whole streptococcal cells, cell walls and protoplast membranes of group A streptococci. There is, however, considerable disagreement as to the situation of the cross-reactive antigen in the bacterial cell or whether or not there is more than one cross-reactive antigen (Zabriskie, 1969; Kaplan, 1969). Human sera with high anti-MAP titres were absorbed with whole cells of M-positive streptococci (types 5, 24 and 30), with the protoplast membranes of a type 49 M-positive strain and also with the homogenates of mammalian tissues reported to bind heart-reactive antibody. These included human heart and skeletal muscle and rabbit heart and skeletal muscle. Complement-fixation tests on sera absorbed in this way showed that anti-MAP had been completely removed from the serum. Absorption with *Staphylococcus aureus* did not absorb the antibody. Absorption

Table 3. *Titre of antibodies to three streptococcal antigens in sera from patients with various streptococcal diseases*

Diagnosis	Number of cases	ASO titre		Anti-DNAse B titre		Anti-MAP titre*	
		Average	Range	Average	Range	Average	Range
Rheumatic fever	20	560	320–984	—	—	143	64–320
Nephritis (following throat infection)	13	473	235–1128	—	—	38	0–160
Nephritis (following skin infection)	17	209	50–675	5100	640–12800	21·25	0–80
Uncomplicated streptococcal infection	20	389	230–730	—	—	17·7	0–120
Normals	20	88	< 50–280	144	< 50–400	9·8	0–40

* Titres of < 10 recorded as 0.

with rabbit kidney gave rather variable results which in general showed a reduction in CFT titre rather than complete removal of the antibody. However, these preliminary experiments were done with relatively large amounts of absorbing agents, and no quantitative assessment has yet been made of the minimum relative quantities of streptococcal cell walls and purified cell membranes needed to remove anti-MAP from the sera (Zabriskie, 1969).

DISCUSSION

The results strongly suggest that the complement-fixing antigen which is present in all M-positive streptococci is closely associated with M-antigen itself. The concomitant increase of specific activity of the two antigens at each stage of the process of purification indicates that they may form part of the same molecule or complex. The existence of a non-specific protein inseparable from M-antigen has previously been suggested by Johnson & Vosti (1968) to explain the cross-reactions observed with some sera when haemagglutination tests with tanned red cells coated with purified M-antigen were used to detect anti-M antibodies in human and rabbit sera. Similar cross-reactions were also observed by Erwa (1968), who used latex particles coated with purified M-antigens. These findings could be explained, in part at least, by the presence of an MAP component of the M-antigen preparations used. Anthony (1970) showed that, in sensitive methods for the quantitative measurement of M-antibody such as the ^{131}I-labelled antigen technique, the most serious limitation is the purity of the antigen. In a study with ^{131}I-labelled type 12 M-protein, purified by ammonium sulphate fractionation and CMC chromatography, he found that the reaction of the antigen with heterologous rabbit sera could be prevented only by the addition of a purified antigen of a heterologous type. Although such cross-reactions are often attributed to insufficient purification of the antigen, our results indicate that increased purification of the type-specific antigen simply resulted in similar purification of the potentially cross-reactive element, MAP. A similar method of purification was used by Beachey, Alberti & Stollerman (1969) in studies of hypersensitivity in the guinea-pig; in these, increasing purity of the M-antigen often resulted in an increased cross-reactivity between M-types.

We have begun to investigate the presence of antibodies to MAP in rabbit antisera. The multiplicity of antibodies to other components of the streptococcal cell, and the presence of anti-complementary activity that is sometimes not removable by pretreatment with complement, makes interpretation of these results difficult. It does appear, however, that antibodies to MAP are present in some but by no means all hyperimmune rabbit sera. Titres up to 320 have been observed.

The ability to detect MAP in group A streptococci by means of a complement-fixation test with an appropriate human serum is useful in streptococcal typing. Many strains, particularly those isolated from skin lesions, cannot be M-typed with the antisera at present available. To find out if they are M-positive has hitherto required the performance of a direct bactericidal test in the blood of at least three normal donors. We find that acid extracts of all apparently untypable strains that are capable of survival in normal human blood will fix complement in the

presence of a human serum that contains anti-MAP. Moreover, the highest dilution of the extract at which complement is fixed with a standard dose of serum gives a good indication of the amount of MAP, and hence the amount of M-antigen, produced by the strain. This could prove useful in selecting a suitable vaccine strain for the production of type-specific antiserum against M-antigens of new types.

The highest titres of antibody to MAP were found in patients with streptococcal infection who had rheumatic fever. The relative titres found in rheumatic fever compared to those seen in sera from other types of streptococcal disease are similar to those described by Zabriskie *et al.* (1970) in their fluorescent-antibody studies of heart-reactive antibody. These workers believe that the streptococcal antigen that is cross-reactive with heart tissue is not M-associated but is present in the streptococcal cell membrane of all group A strains. On the other hand Kaplan (1966, 1969) suggests that there are at least two cross-reactive streptococcal antigens and favours a close association of one of these (CR 1) with M-antigen.

Our preliminary absorption experiments also suggest a similarity of anti-MAP to heart-reactive antibody. Should more carefully controlled quantitative experiments confirm a relationship, then the undoubted association of the MAP antigen with M protein may prove to have some bearing on the divergent views concerning heart-reactive antibody.

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The Australia (hepatitis-associated) antigen amongst heroin addicts attending a London addiction clinic

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SUMMARY

Thirty-three of 72 heroin addicts attending a recognized clinic for drug addition had a history of jaundice, but in only five was the serum positive for hepatitis-associated antigen (HAA) when examined by immunodiffusion, immunoelectro-osmophoresis and complement fixation. Two of these were repeatedly positive over an 8–12 month follow-up period and liver biopsy showed chronic persistent hepatitis. A third later developed acute hepatitis. A study of the injection habits suggested that the present low incidence of HAA and the decrease in number of cases with jaundice was probably related to the provision of free disposable syringes by the clinic since it was opened in 1968.

INTRODUCTION

Since the first report by Steigmann, Hyman & Goldbloom (1950) of an outbreak of hepatitis amongst narcotic addicts, there have been numerous reports suggesting that the infection is acquired through the communal use of syringes and needles (reviewed by Zuckerman, 1970). Although serum hepatitis is now known to be specifically associated with the presence of Australia (hepatitis-associated) antigen (HAA) in the blood, the sensitivity of the currently available techniques for its detection does not provide an absolute index of infection (*Bulletin of the World Health Organization*, 1970). It is uncertain whether the antigen represents incomplete virus particles, an aggregate of protein subunits, excess production of unstable virus-like particles or a specific protein produced by cells infected with the serum hepatitis agent. Nevertheless, the availability of tests for this antigen has provided the means for studying and re-evaluating the epidemiology of serum hepatitis. In this paper we report a survey of 72 heroin addicts attending one of the recognized centres for drug addition (the St Giles' Clinic, Camberwell), with particular reference to the incidence of HAA, the occurrence of hepatitis and the injection habits of the addicts.

PATIENTS AND METHODS

The 49 male and 23 female addicts seen had been addicted for periods of 1–15 years. All had taken heroin regularly, although some had recently been weaned to physeptone or methadone. The addicts were questioned specifically for a history of jaundice and about their injection habits. Liver function tests were performed whenever sufficient blood could be obtained, and when abnormal, a complete physical examination was performed.

Serum was examined for HAA by immunodiffusion (Zuckerman & Taylor, 1969), by immunoelectro-osmophoresis (Zuckerman & Taylor, 1970) and by a micro-titre complement-fixation test (Taylor, 1970). The laboratory reference antigen gave a reaction of immunological identity with other known Australia (hepatitis-associated) antigens (*Bulletin of the World Health Organisation*, 1970).

RESULTS

None of the 72 addicts were jaundiced at the time of the survey, although 33 (46%) gave a history of jaundice in the past. An analysis of the years in which these attacks of jaundice had occurred showed a peak incidence in 1967, and since 1968 when the clinic was started the frequency has declined (Fig. 1). This may have

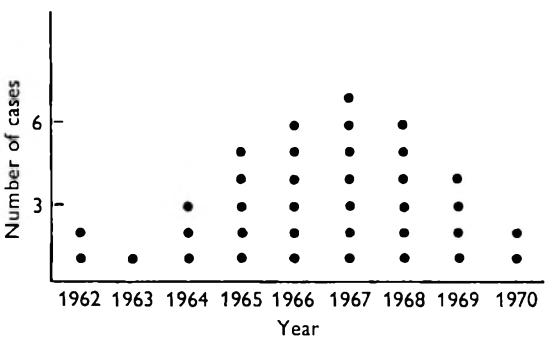

Fig. 1. The number of cases with jaundice each year.

been related to the provision in the clinic of free disposable syringes, ampoules of sterile water and advice on syringe hygiene, for the incidence of previous jaundice was significantly higher in those who admitted sharing syringes with other addicts (Table 1). It was also higher in those who used water from the lavatory pan to make up injections rather than tap or sterile water and in those who did not cleanse the skin before injection, although in neither instance did the difference reach statistical significance.

Liver function tests were carried out in 53 addicts which included 28 of the 33 with a past history of jaundice. None had a raised serum bilirubin level, but four had an elevated serum alkaline phosphatase and in 17 (32%) the serum aspartate aminotransferase (SGOT) was moderately raised with values up to 122 mU./ml. The serum gamma globulin was greater than 1·3 g/100 ml., the upper limit of

normal, in 34 (83 %) of the 41 patients in whom it was estimated. The highest gamma globulin level encountered was 3·1 g/100 ml., in a patient who had previously been jaundiced but whose serum aspartate aminotransferase was only 25 mU./ml. No relationship could be detected between these abnormalities in liver function tests and a previous history of jaundice.

Table 1. *The frequency of a history of jaundice in the* 72 *addicts related to injection habits*

Injection habits		Percentage of total number of addicts	Percentage of addicts in each group with a history of jaundice
Sharing of syringes	Yes	71	52
	No	29	26
Source of injection water	Lavatory pan on occasion	45	62
	Always tap or sterile	55	47
Cleansing of skin before injection	No	83	50
	Yes	17	30

Table 2. *Clinical details of the five addicts with HAA in their sera*

Case	Syringe sharing	Previous jaundice	Serum aspartate aminotransferase (mU./ml.)	Persistence of HAA	Liver biopsy
5	Yes	No	24	8 months	Chronic persistent hepatitis
19	Yes	Yes (1967)	55	12 months	
31	Yes	No	85	4 months	Acute hepatitis (3 months later)
35	Yes	Yes (1966)	20	—	—
44	No	No	24	—	—

Australia (hepatitis-associated) antigen

This was found in the sera of five patients. In three the positive result was obtained in the immunodiffusion test, but in the fourth patient HAA was detected by immunoelectro-osmophoresis, and in the fifth by complement fixation alone. The presence of HAA was confirmed in all five patients by electron microscopy.

Of the five patients, two gave a history of previous attacks of jaundice (Table 2). One of these (Case 19) had recently given up drugs and remained clinically well during the subsequent follow-up period of 12 months, although his serum remained consistently positive for HAA. Liver function tests were virtually normal, but a liver biopsy showed the histological appearances of chronic persistent hepatitis. These were also seen in the liver biopsy of Case 5 who, unlike the previous patient, was not aware of jaundice in the past. His liver function tests were normal at the time of survey and remained so until his death eight months later from an overdose, his serum during this time being repeatedly positive for HAA. Another patient

(Case 31) had no previous history of jaundice and had normal liver function tests when his serum was first found to be positive for HAA in February 1970. Three months later he developed acute hepatitis with jaundice and the characteristic histological changes on liver biopsy. From this, he appeared to make a complete recovery and HAA disappeared from the blood.

DISCUSSION

Nearly half of these addicts had had jaundice, but in only five (7%) was HAA detected in the serum. This cannot be attributed to use of relatively insensitive techniques, since we employed in parallel immunodiffusion, immunoelectro-osmophoresis and the complement-fixation test, which in our hands is very sensitive. Some of our patients may have become immune to hepatitis as a result of previous exposure. Immunity to second attacks of hepatitis has been reported in human volunteer experiments by MacCallum (1953) and Giles, McCollum, Berendtson & Krugman (1969). In none of the present patients were we able to detect antibody to HAA in the serum by the immunodiffusion technique. Immunity may, however, depend on cell-mediated mechanisms. In fact, three of our patients claim to have had more than one attack of jaundice. Although it has been suggested that the drugs and other ingredients used for dissolving them may have a direct hepatotoxic effect (Marks & Chapple, 1967), this view is not supported by epidemiological evidence in outbreaks of hepatitis (Bewley, Ben-Arie & Marks, 1968), or by experimental data (Brooks *et al.*, 1963; Gorodetzky, Sapira, Jasinski & Martin, 1968). There was a significant association in our patients between a history of jaundice and the sharing of syringes. It appears likely that the sterile syringes supplied by the clinic since it opened in 1968, together with instruction concerning hygiene, have resulted in a lower rate of transmission of serum hepatitis.

Nevertheless, in two of the five patients found to have HAA at the time of the survey, the antigen persisted over long periods of time. This was associated with histological changes of chronic persistent hepatitis. Norris & Potter (1965) found similar histological changes with infiltration of lymphocytes in the portal tracts in 27 of 36 addicts examined, but this unfortunately was before tests for HAA were generally available. To what extent chronic persistent hepatitis is the result of persistence of HAA is uncertain at present. Becker, Scheuer, Baptista & Sherlock (1970) have suggested that the prognosis of chronic persistent hepatitis is good. Their patients, however, were not addicts. Recently, Tamburro, Rajan & Leevy (1971) have reported a follow-up study of 104 patients including 63 addicts, with non-epidemic hepatitis. Four addicts developed cirrhosis and ten fibrosis. In six of these the serum was positive for HAA. Cirrhosis was seen to follow hepatitis in addicts in whom HAA was not detected, and malnutrition and alcoholism as well as persistent antigenaemia with repeated re-inoculation are probably all important factors contributing to its development. One-third of the patients in the present series had raised serum aspartate aminotransferase levels, and in over two-thirds the serum gamma globulin was elevated. Bewley, Ben-Arie & Marks (1968) reported a similar frequency of abnormal liver function tests in a series of 254 non-jaundiced

addicts. Histological abnormalities in the liver might well be apparent in these patients if liver biopsies were performed.

Serum hepatitis can be transmitted not only parenterally, but also by the oral route (Krugman, Giles & Hammond, 1967; Krugman & Giles, 1970) and persisting infection in the addict can be spread to family contacts. One of our patients was potentially infectious for at least 3 months before the onset of clinical hepatitis, and the two with chronic persistent hepatitis had HAA in their sera for 8 and 12 months respectively. Although the incidence of HAA in the clinic as a whole is low, it would seem worth while to screen all drug addicts when they register initially and thereafter at regular intervals.

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Neuraminidase and resistance to vaccination with live influenza A2 Hong Kong vaccines*

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SUMMARY

Thirty-seven volunteers were inoculated intranasally with living attenuated influenza A2 viruses. Rising titres of circulating antineuraminidase (AN) were detected in 14 of 17 infected volunteers. AN was also found in nasal secretions. Statistical analysis showed that there was a correlation between the titres of haemagglutination-inhibiting antibody (HI) and AN in nasal washings, and between AN in blood and washings. Resistance to infection could be predicted from antibody titres in 29 of 37 volunteers and blood AN alone predicted the outcome of 25 volunteers.

INTRODUCTION

It has been known for some time that vaccination with living attenuated influenza A virus strains protects against infection with the same serotype of virus either by re-exposure to the vaccine strain or by natural exposure in epidemic conditions. It has also been shown that infection with unattenuated virus stimulates circulating antibodies and also neutralizing (N) and haemagglutination-inhibiting (HI) antibody in the nasal secretion (Mann *et al.* 1968). Antineuraminidase (AN) appears in serum and secretion after vaccination with live influenza B virus (Downie, 1970); but more information is needed about the importance of circulating and local antibody in resistance to influenza virus infections. We have therefore undertaken studies in which influenza A vaccines were administered to normal subjects in isolation and serum and nasal antibodies were measured.

MATERIALS AND METHODS

Volunteers in isolation at the Common Cold Unit, Salisbury, were inoculated with attenuated influenza virus vaccines using methods previously described (Tyrrell, 1963). They were inoculated in two trials, nos. 74 and 76, which took place at the end of January and the beginning of April 1970.

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Specimens

Nasal washings were collected before inoculation and 7 days after, using about 10 ml. of sterile phosphate buffered saline (PBS). These were shown to be free of blood by the Hemastix test, were dialysed against distilled water, freeze-dried, and reconstituted in 1/10 the original volume of PBS. Blood was collected before inoculation and both 7 days and 3 weeks later. Serum was separated aseptically. These specimens were stored at $-20°$ C.

Nasal washings were also collected on the 2nd, 3rd and 4th days after inoculation, for virus isolation by allantoic inoculation of embryonated eggs.

Antibody titrations

Haemagglutination inhibition (HI) tests

To destroy non-specific inhibitors sera were treated with 5 volumes of cholera filtrate and nasal secretions with an equal volume. Sera were inactivated and titrated with virus in the form of allantoic fluid; secretions were titrated against viruses treated with tween 20 (final dilution 1/20,000) and equal parts of ether; versene saline was used as diluent in the titrations to prevent the effect of neuraminidase on cells.

In all cases 4 units of virus were mixed with each dilution of serum or nasal secretion and the mixtures were held at room temperature for 30 min.; 0·5% human red cells were then added.

Antineuraminidase (AN) assays

Neuraminidase activities were assayed by a modification of Warren's method as described by Webster & Laver (1967) using fetuin as substrate. Assays of antineuraminidase activity in sera and nasal washings were performed essentially as described by Schild & Newman (1969), but with certain modifications to increase the sensitivity and specificity of the test:

(*a*) A reduced amount of viral neuraminidase was used, the concentration of virus used as a source of neuraminidase was adjusted so that after incubation with excess substrate for 16 hr. at 37° C. at pH 5·9 the amount of *N*-acetyl neuraminic acid released per 0·05 ml. of virus was 10–15 μg.

(*b*) Virus and serum (or nasal washing) dilutions were incubated at room temperature for 3 hr. during the enzyme neutralization reaction.

(*c*) The source of neuraminidase was a recombinant influenza virus, FPV-HK (kindly provided by Dr D. McCahon, National Institute for Medical Research, London), between fowl plague virus and A2/Hong Kong/68 containing neuraminidase of the A2 virus and haemagglutinin of the fowl plague virus. The use of the recombinant virus, since it contained haemagglutinin unrelated to that of the human Hong Kong virus, or other human influenza A viruses, avoided the possibility that anti-haemagglutinin antibody might produce non-specific inhibition of enzyme activity by 'steric hindrance' (Easterday, Laver, Pereira & Schild, 1969; Schild, McCahon & Kendal, 1970). The titres of antineuraminidase were expressed as the dilution of serum (or washing) inhibiting 50% of enzyme activity.

Immunodiffusion tests

Immunodiffusion was performed using concentrates of A 2/Hong Kong/68 virus disrupted by sodium dodecyl sulphate as described previously (Schild & Pereira, 1969; Schild, Winters & Brand, 1971). Sera with high titres of antineuraminidase activity were found to produce precipitin lines corresponding to virus neuraminidase. However, this test was too insensitive to detect antineuraminidase antibody at the low levels found in nasal secretions.

Vaccines

The three vaccine virus strains were antigenically identical and were developed at the Common Cold Unit and produced there or else prepared at the State Institute for Viral Preparations in Moscow, U.S.S.R. The strains were derived from A 2/Hong Kong/1/68 and A 2/Istra/10/69. Their effects are described in detail elsewhere (Beare, Maassab, Slepushkin & Hall, 1971), but since their behaviour in human volunteers was generally similar we have combined the results. Volunteers received 10^5 EID 50 of virus as nasal drops.

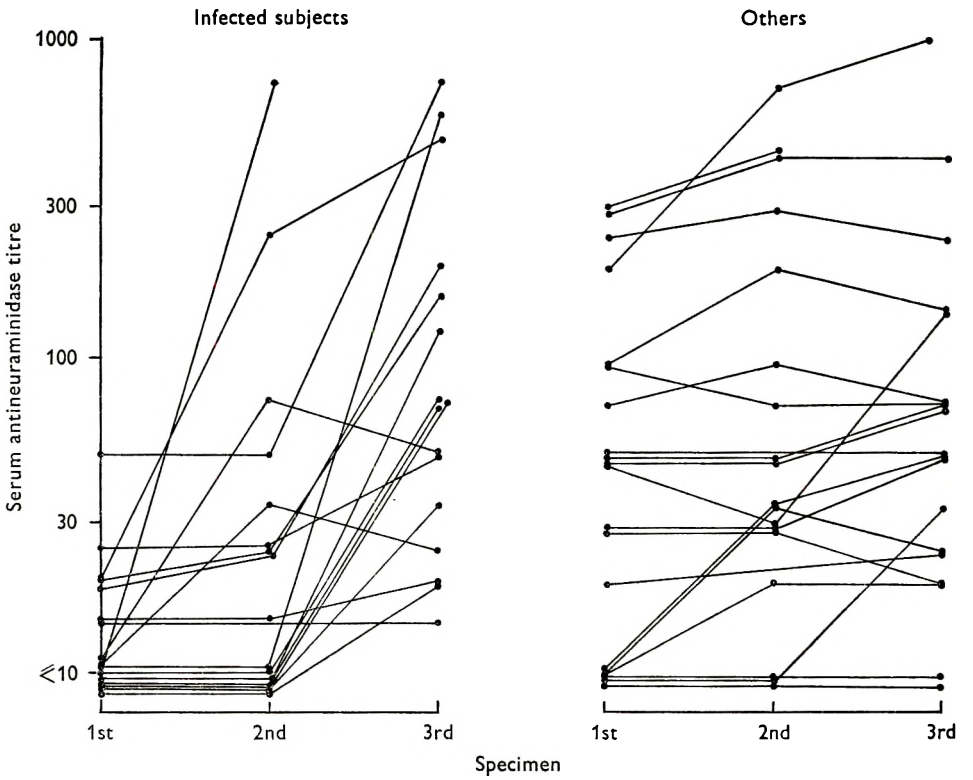

Fig. 1. Titre of serum antineuraminidase antibody. The left panel shows the results in volunteers who were shown to be infected by rising titres of HI antibody with or without virus isolation. The right-hand panel is the results in those who were uninfected by these criteria. The first titre was that immediately before vaccination. The second was collected at the Unit 7 days after vaccination and the third about 3 weeks after the volunteers had returned home.

RESULTS

For the first analysis volunteers were regarded as infected if virus was isolated or if a rising titre of HI antibody occurred and we considered first the response of circulating antibody and especially AN in infected and uninfected volunteers.

A substantial number of volunteers had circulating HI at the time of challenge – the low titres may have been due to infection with previous Asian strains, but the high titres probably resulted from previous infections with HK strains. As was expected antibody titres rose after infection in many cases, but there was not

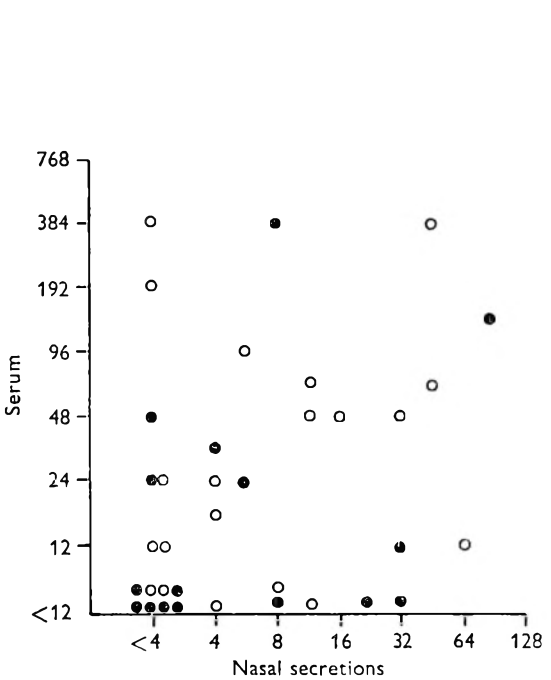

Fig. 2

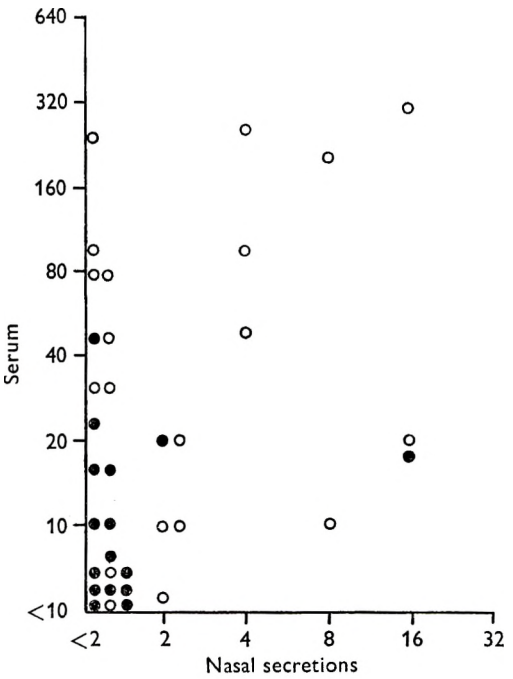

Fig. 3

Fig. 2. Correlation between the titre of HI antibody in serum and nasal secretion of 36 subjects before vaccination. The open symbols show volunteers who resisted infection and the closed those who became infected as judged by HI antibody rises and virus isolation.

Fig. 3. A similar diagram to Fig. 2, showing the relationship between titres of AN antibody in the two compartments.

complete concordance between the occurrence of rising titres and virus isolation; in particular a few subjects yielded virus in one of three specimens and showed no circulating antibody response.

The results of serum AN tests are summarized in Fig. 1. Sera with high AN titres produced precipitin lines corresponding to AN indicating that the AN activity was due to antibody. Fig. 1 shows that the volunteers who became infected had lower titres on the whole than those who were not infected – as judged by HI rises and/or virus recovery. Eleven of 17 who were infected had AN titres of under 20 as against 5 of 20 who were not infected. AN rises occurred in 14 of 17 infected

volunteers but also in 5 of 20 volunteers in the 'uninfected' group – those which occurred early might have represented a response to the antigen in the inoculum but it is more likely that they represented limited infections which were not detected in the other tests used. The titres after vaccination of the subjects who were infected were similar to those of the subjects who resisted infection by the vaccine.

Nasal washing antibodies

The titres of HI in nasal washings were much lower than those in serum and the relation between these and serum HI are shown in Fig. 2. There was no close correlation between the amounts of antibody at the two sites, in particular high titres were found in one and low in the other. In the five days following vaccination there were in the nasal washings rising HI titres in seven subjects and AN titres in three.

In Fig. 3 is shown the relationship between the AN level in serum and the nasal washings and here again the correlation was not particularly close. There seemed, nevertheless, to be a general correlation between the AN and HI titres in the secretions, although a number of specimens with HI titres had no AN activity.

Statistical analysis showed that the titres of each antibody were positively correlated with those of each of the other three; the correlation coefficient between titres of HI and AN in washings was +0·47 (significant at 1% level) and between titres of AN in blood and washings was +0·40 (significant at 5% level). The other coefficients were not significantly different from zero.

Antibody and resistance to infection

Inspection of Fig. 2 showed that infection occurred in the presence of antibody; however, infection was infrequent (5 of 15) if both nasal and serum HI were detectable (titre of 1/4 or 1/12 or greater) and more frequent (6 of 8) if both were undetectable, and the same applies to AN antibody (Fig. 3). It was striking that only 2 of 13 volunteers with detectable nasal AN antibody were infected whilst 14 of 23 volunteers without such antibody were infected.

The simple analyses used so far were not able to show whether resistance was really related to all the antibodies detected or whether one was more important than others; apparent effects might have been due to correlations between the presence of one type of antibody and another. Therefore a more thorough analysis was performed.

After ascribing a variable with value 1 to those subjects with a positive reaction to the challenge and 0 to those with a negative reaction, and transforming titres to logs, discriminant analyses were carried out by regressing this variable on the titres of each antibody in turn. Titres recorded as 'less than' were given the next lower titre in the dilution series used. The slope was negative for each antibody considered on its own, indicating that those subjects with a low titre of an antibody were more susceptible to influenza than those with a high titre of the same antibody. The separation between the group of subjects who contracted influenza when challenged and the group who did not was most marked when the titre of AN

in the blood was used as the discriminating factor (variance ratio significant at 1% level). Regressing on the titre of AN in the nose the variance ratio was significant at the 5% level; the titre of HI in the blood gave a non-significant variance ratio, and that for the titre of HI in the nose was less than 1.

The titres of AN in the blood were lower for trial 76 than they were for trial 74. For trial 74 regression on the titre of AN in the blood gave a variance ratio significant at 0·1%. When trial 76 was considered separately regression on the titre of AN in the nose gave the best separation between the groups, with the titre of HI in the blood second best.

The extra reduction in the residual sum of squares obtained by regressing on all four variables compared with regressing only on the titre of AN in the blood was not significant. The one missing AN titre was replaced by the mean titre. A prediction of the result of the challenge based on the multiple regression would have classified correctly 29 of the 37 subjects, whereas prediction by the titre of AN in the blood would classify correctly 25 of them.

It was concluded that of the four factors a high titre of AN in the blood contributed most to resistance to infection – there was no evidence that high titres of anti-haemagglutinin, in addition to this, had any effect in increasing the resistance.

The data were also analysed by using the titres of antibody to classify the subjects, and applying the logit transformation ($z = \frac{1}{2} \log p/q$) to the proportion of subjects in each class who contracted influenza. The logit was then used as the dependent variable in a regression on the titres of the antibodies. Maximum likelihood estimates of the parameters led to the same conclusions as the above.

DISCUSSION

The first point of interest in this study is that live influenza vaccine like natural infections and administration of killed vaccine stimulated AN production (Kilbourne, Christenson & Sande, 1968; Schild & Newman, 1969). It has recently been shown in other studies that one of our Salisbury live vaccine strains also stimulates AN in the nasal secretions (Downie & Stuart-Harris, unpublished; Tyrrell *et al.*, unpublished) as did a live influenza B vaccine (Downie, 1970). There is abundant evidence from early work and from our own studies in volunteers that there is a general correlation between the titre of circulating HI antibody and resistance to infection with the same serotype of influenza virus, but it could not be assumed that AN protects.

Evidence from some studies suggests that the neutralizing antibody content of nasal secretion determines almost entirely whether a subject becomes infected or not – examples are the work of Smith, Purcell, Bellanti & Chanock (1966) with parainfluenza 1 and Perkins *et al.* (1969) with a rhinovirus. On the other hand, in other studies, namely with parainfluenza 2 (Tremonti, Lin & Jackson, 1968) and influenza B (Downie & Stuart-Harris, 1970), it seemed that resistance was, to a considerable extent, correlated with high titres of circulating antibody.

The application of discriminant analysis to the results of challenge can, we believe, help to resolve the complicated situation, although it cannot explain the mechanism

of the relationship detected. The poor correlation between resistance to infection and the results of HI tests may partly result from these tests being less reliable than the AN test, rather than that AN itself has more effect. It would now be interesting to test for neutralizing antibody also, but unfortunately many nasal specimens are exhausted and a proper analysis would therefore not be possible. Nevertheless it seems likely that circulating AN mediates immunity to infection – it may well leak out into the respiratory tract and limit the spread of virus there as may be seen in experimentally infected animals and in tissue cultures infected with influenza virus (Kilbourne, Laver, Schulman & Webster, 1968).

The decline in the titre of circulating AN between the first and second trial is probably a reflexion of the rather rapid loss of this type of antibody previously observed after natural infections with influenza (Schild & Newman, 1969). This is a reminder of the fact that the immune status of the population is always changing, and although AN may have an important effect on immunity shortly after an epidemic, it is probable that at other times other types of antibody might make the major contribution.

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Vaccination against rubella of susceptible schoolgirls in Reading

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SUMMARY

This study of 724 13-year-old schoolgirls in the County Borough of Reading showed that approximately 25% were susceptible to rubella. 96·1% of the 129 seronegative girls and significant numbers of girls with low rubella HAI antibody titres responded to subcutaneous vaccination with Wistar RA. 27/3 rubella vaccine. The incidence of most reactions after vaccination was similar in those who responded to vaccine and those who were initially immune but did not develop rising antibody titres, but rash, lymphadenopathy and headache occurred significantly more frequently in the susceptible group.

INTRODUCTION

It has been estimated that an epidemic of rubella caused the birth of between 10,000 and 20,000 infants with congenital rubella malformations in the United States between 1964 and 1966 (Cooper & Krugman, 1966). In the United Kingdom some 200 infants with rubella malformations are thought to be born annually in non-epidemic years (Dudgeon, 1969). In addition, an unrecorded number of abortions are carried out each year as a result of rubella or exposure to rubella during pregnancy.

Vaccination against rubella is unique in immunizing practices. Vaccination is aimed to give protection, not to the vaccinee, but to any children that may be conceived. Furthermore, it has not been shown that vaccine virus is devoid of embryotoxic and teratogenic properties. The potential teratogenicity of vaccine virus has led to 11- to 13-year-old schoolgirls in whom the risk of pregnancy is slight, being chosen as the prime group for vaccination against rubella in the United Kingdom (Godber, 1970).

This report describes the results of screening and subsequent vaccination of schoolgirls in the County Borough of Reading. The study was designed to:

1. Determine the immunological status of 13-year-old schoolgirls against rubella as evidenced by haemagglutinating inhibiting (HAI) antibody titres.
2. Identify rubella-susceptible schoolgirls.

3. Determine the serological response to subcutaneous administration of Wistar RA. 27/3 rubella vaccine.

4. Determine the incidence of reactions to rubella vaccine.

PLAN OF STUDY

Thirteen-year-old schoolgirls in State schools in the County Borough of Reading were offered serological screening for rubella HAI antibody, and vaccination if necessary. Formal consent was secured from parents or guardians. Subsequently, seronegative girls and girls with low rubella HAI antibody titres were vaccinated. Schoolgirls with low titres were vaccinated in the light of reports which have suggested that subjects with low antibody titres may respond to vaccination with fourfold or greater increases in titre (Dudgeon, Marshall, Peckham & Hawkins, 1969).

During the 21-day post-vaccination period girls completed a calendar record card which listed symptoms of sore throat, rash, pain and shivering attacks (fever). They were bled 6 weeks after vaccination and HAI antibody again titrated.

Serology

Rubella HAI antibody titrations were carried out as described by Draper & Kelly (1969), at The Wellcome Research Laboratories. In this technique, Whatman No. 3 chromatography paper disks are saturated with capillary blood from a finger prick, and later eluted for antibody titration.

Vaccine

Lot No. AR. 5/1 Wistar RA. 27/3 rubella vaccine was administered subcutaneously. This vaccine was prepared at the Wellcome Research Laboratories, Beckenham, at a titre of $10^{2.53}$ TCID 50/dose.

RESULTS

Serological results

Results were obtained from 724 of 787 (92%) 13-year-old schoolgirls offered a determination of rubella antibody. The response of parents to offers of pre-vaccination screening for rubella immunity and vaccination if necessary was high – over 90% consented to both procedures (Table 1).

The distribution of HAI antibody titres of these girls is shown in Fig. 1. HAI antibody titres of 1/40 or less were found for 285 girls (39·4%). A total of 255 girls were vaccinated, 232 with titres of 1/40 or less, 19 with titres of 1/80 and four with titres of $> 1/80$. Some girls were not vaccinated because they were away from school, while vaccine was deliberately withheld from three girls, one with diabetes mellitus, one with petit mal, and one with a past history of thrombocytopenic purpura. No significant differences were found between rubella HAI antibody titres at screening and those obtained during re-titration of pre-vaccination blood with the 6-week post-vaccination sample. Pre-vaccination and 6-week post-

vaccination HAI antibody titres are shown in Table 2. A sero-conversion rate (fourfold or greater increase in antibody titre) of 96·1 % resulted from vaccination of those with pre-vaccination titres of < 1/10. However, the seroconversion rate decreased with increasing pre-vaccination titres (Table 3). Thus 8 of 20 (40 %) and 14 of 52 (26·9 %) girls with pre-vaccination titres of 1/20 and 1/40 respectively developed fourfold or greater increases in antibody. No such effects were seen in 19 girls with pre-vaccination titres of 1/80.

Table 1. *Results of offer of serological screening for immunity to rubella and vaccination if required*

Total number of consent forms issued	...		787	—
Consent given to blood test	730	92·7 %	—	—
Consent refused to blood test	39	5·0 %	—	—
Consent given to vaccination	717	91·1 %	—	—
Consent refused to vaccination	52	6·6 %	—	—
Total number of consent forms returned	769	—	769	97·7 %
Consent forms NOT returned	—	—	18	2·3 %

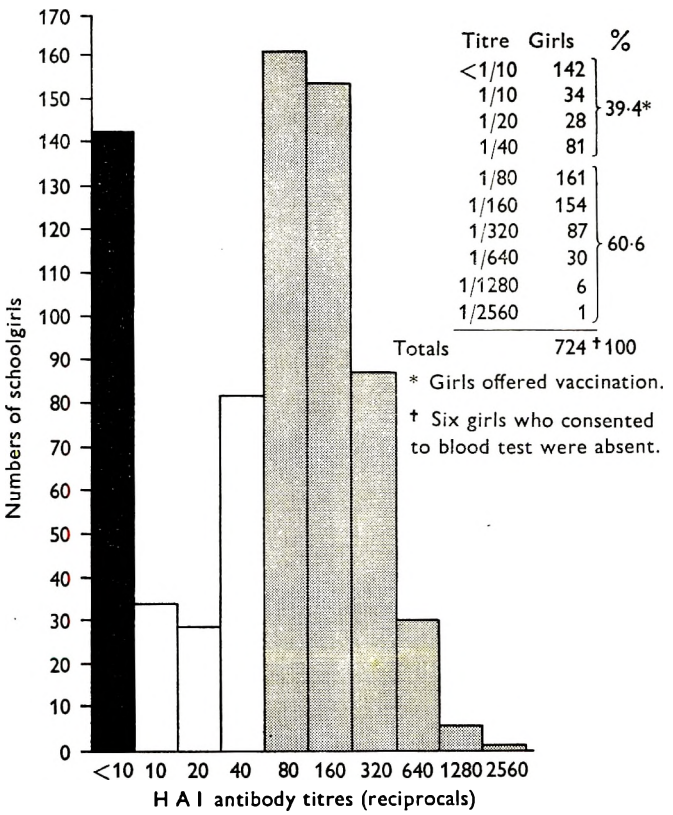

Fig. 1. Pre-vaccination rubella antibody distribution (13-year-old girls).

Table 2. *Serological responses to Wistar RA. 27/3 rubella vaccine administered subcutaneously to schoolgirls in Reading*

No. of schoolgirls	Pre-vaccination HAI titre	Six-week post-vaccination HAI antibody titres							
		< 1/10	1/10	1/20	1/40	1/80	1/160	1/320	1/640
129	< 1/10	—	1	4	25	51	34	14	—
31	1/10	—	0	1	6	10	10	4	—
20	1/20	—	—	4	8	4	2	2	—
52	1/40	—	—	—	19	19	10	2	2
19	1/80	—	—	—	2	13	4	—	—
4	> 1/80	—	—	—	—	—	3	—	1

Table 3. *Pre-vaccination titres and seroconversion rates*

Pre-vaccination HAI antibody titres	No. of children	Number showing fourfold increase in antibody titre
< 1/10	129	124 (96·1 %)
1/10	31	30 (96·8 %)
1/20	20	8 (40 %)
1/40	52	14 (26·9 %)
1/80	19	0
> 1/80	4	0
Totals	255	176

Reactions

Of the 255 children who were vaccinated, 252 completed their record cards. According to their serological response to vaccine, children were divided into two groups:

Group R (vaccine responders), included children with a fourfold or greater increase in antibody titre between pre- and post-vaccination titres; and

Group N (vaccine non-responders), the remainder who failed to show a fourfold increase.

The distribution of children into groups R and N is shown in Table 4, and the incidence of reactions between the two groups compared in Table 5. The incidence of sore throat, reported to be a common symptom following vaccination against rubella, was not found to be different in our R and N groups. While the incidence of the joint symptoms between the groups is little different, the two cases of obvious swelling of the knee joint in R group children are likely to be vaccine induced. Analysis of daily reports of symptoms shows that lymphadenopathy was most commonly reported at about the 11th day after vaccination, and was still present when the record card finished on the 21st day. Rash occurred most commonly on the 13th to 16th days after vaccination (Table 6). Children suffering from psoriasis, eczema, asthma and hay fever were vaccinated without complication.

Table 4. *Pre-vaccination antibody titres of 'R' and 'N' Group Girls, and serological response to vaccination*

Pre-vaccination HAI antibody titres	Group R*	Group N†
< 1/10	121	5
1/10	30	1
1/20	8	12
1/40	14	38
1/80	—	19
> 1/80	—	4
Total no. of children	173	79

* Group 'R' – (Vaccine responders). Girls showing a fourfold or greater increase in HAI antibody titre after vaccination.

† Group 'N' – (Vaccine non-responders). Girls not showing a fourfold increase in HAI antibody titre after vaccination.

Table 5. *RA. 27/3 rubella vaccine – comparison of reactions reported in schoolgirls between groups R and N*

Reactions	Group 'R' (total 173)*		Group 'N' (total 79)†	
No reactions	64	37·0 %	35	44·3 %
Sore throat	63	36·4 %	32	40·5 %
Fever	20	11·6 %	13	16·5 %
Rash	25	14·5 %	3	3·8 %‡
Joint or muscle pain	16	9·2 %	9	11·4 %
Coryza	17	9·8 %	9	11·4 %
Stomach pain	23	13·3 %	7	8·7 %
Post-injection pain or inflammation	7	4·0 %	2	2·5 %
Headache	34	19·6 %	5	6·3 %‡
Pain in neck	5	2·9 %	1	1·3 %
Other	4 (Earache; Earache and conjunctivitis; Vomiting; Nausea)		3 (Earache; Malaise; Nausea)	
Lymphadenopathy	13	7·5 %	0	—‡
Break-down of subjects showing joint involvement	Pains in arm	4		4
	Pains in arm and hands	2		—
	Pain in legs	3		1
	Pain in knees	1		—
	Swelling in knees	2		—
	Pain in hips	2		—
	Generalized	0		3
	Other	2		1

* Group 'R' – (Vaccine responders). Girls showing a fourfold or greater increase in HAI antibody titre after vaccination.

† Group 'N' – (Vaccine non-responders). Girls not showing a fourfold increase in HAI antibody titre after vaccination. ‡ – $P < 0{\cdot}05$.

Table 6. *Comparison on a daily basis of reactions reported by group 'R' children and group 'N' children*

Symptom	Group	1+2	3+4	5+6	7+8	9+10	11+12	13+14	15+16	17+18	19+20
Rash	R	2	2	7	3	1	6	26	23	7	8
	N	0	0	2	2	2	0	0	0	0	0
Lymphad-enopathy	R	0	1	2	2	6	20	19	18	18	18
	N	0	0	0	0	0	0	0	0	0	0
Sore throat	R	22	23	28	21	19	23	32	23	10	10
	N	8	10	10	16	14	9	9	9	4	9
Headache	R	13	12	12	9	8	16	12	9	8	2
	N	2	2	1	1	1	0	0	0	2	0
Pain in neck	R	1	3	2	0	2	4	2	2	2	2
	N	2	0	0	0	0	0	0	0	0	0
Abdominal pain	R	2	3	3	10	4	7	6	2	1	4
	N	6	3	1	1	2	3	1	1	1	0
Joint in-volvement	R	2	4	1	3	3	1	3	4	4	5
	N	6	3	1	1	2	3	1	1	1	0

Group R (vaccine responders). Total number of children, 173.
Group N (vaccine non-responders). Total number of children, 79.

DISCUSSION

In this study the use of Whatman No. 3 chromatography paper disks proved highly satisfactory in contrast to experiences in an earlier study (Rowlands & Gatherer, 1970) where difficulties were encountered in obtaining full saturation of disks in a proportion of 15-year-old girls.

HAI antibody determinations are widely carried out as an index of immunity to rubella. It is clear, however, that subjects with low antibody titres may respond to subcutaneous vaccination. Similarly, there are an increasing number of reports which show that subjects with low titres of antibody induced by vaccination are open to re-infection with rubella virus (Horstmann *et al.* 1970; Wilkins, Leedom, Portnoy & Salvatore, 1969; Meyer *et al.* 1969). The critical point is whether or not subjects re-infected after natural infection or vaccination develop viraemia with consequent fetal risk. Viraemia has not been demonstrated in such instances but the evidence available is limited. At present therefore it seems unreasonable not to vaccinate girls with low antibody titres. Thus, if those who respond to vaccine are susceptible to rubella, 25% of this population are not immune to the natural infection. Therefore, if schoolgirls with low HAI antibody titres cannot be presumed to be immune, this forms a point in favour of vaccination of all 11- to 13-year-old girls, since there may be difficulties in determining the degree to which low-titre seropositive girls should be vaccinated, in view of the differences in the sensitivity of HAI antibody titrations between laboratories. Although approximately 25% of girls were susceptible to rubella in this study, it was necessary for approximately 40% to be vaccinated to ensure that no susceptible girl with a low titre was left unprotected.

Initial cost analysis suggests that serological screening and vaccination of susceptibles alone would be less expensive than vaccination of all 11- to 13-year-old girls. This study has shown that screening and vaccination of rubella susceptibles is a practical procedure, of the type normally carried out by the School Health Service. It may be that at the present time laboratory facilities are not sufficient to deal with screening on a national scale, but it is clearly inadvisable for 60% of schoolgirls to be vaccinated against rubella unnecessarily.

The incidence of reactions in this study is high. Clearly any sensitive subjective daily reporting system inevitably collects many intercurrent symptoms, while related clinical signs may be under-reported. Nevertheless, this method of recording reactions is convenient, clearly effective, and appears to be reliable in recording critical factors of patient disease.

Although unrelated to the method of recording reactions, difficulties were experienced in the assessment of joint symptoms. It seems clear that non-specific muscular pain has been included as joint involvement in both R and N groups.

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Aspects of influenza C virus replication

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SUMMARY

Influenza C virus antigen could be detected by immunofluorescence in chick amniotic epithelium 4 hr. after inoculation with undiluted amniotic fluid. Antigen could be detected in the nucleus of rhesus monkey kidney tissue cultures 10 hr. after infection. Only cytoplasmic fluorescence was observed 17–21 hr. after infection.

The pattern of replication was similar to that reported for group I myxoviruses, indicating that the virus is correctly classified as an influenza virus.

INTRODUCTION

Taylor (1949) isolated a strain of influenza virus 1233, which appeared to be antigenically unique. Francis, Quilligan & Minuse (1950) isolated a JJ strain which proved to be identical with 1233, and they suggested that these strains should be designated influenza C virus. Quilligan, Minuse & Francis (1951) and, later, Minuse, Quilligan & Francis (1954) reported in detail on the properties of influenza C virus, which differ from those of A and B viruses to a greater extent than the properties of A and B viruses differ from each other.

We investigated the replication of influenza C virus in rhesus monkey kidney cells and in the amnion of chick embryos by immunofluorescent methods to discover whether the pattern resembles that reported for influenza A and B (Watson & Coons, 1954, and many subsequent workers) or that reported for myxovirus group 2 and paramyxoviruses (Wheelock & Tamm, 1959; Traver, Northrop & Walker, 1960).

MATERIALS AND METHODS

Viruses

The following two strains were used: influenza C/Paris/1/67 and influenza A2/Hong Kong/1/68.

Conjugate

SWAR, swine anti-rabbit globulins, obtained from Nordic Diagnostics, Fraburg Ltd, was used at a 1/20 dilution.

* Supported by a grant from the Medical Research Council. Part of this work was presented in her M.Sc. thesis at the University of Birmingham, 1969.

Antisera

The following were used.

(1) A/4/69. Influenza C antiserum produced by inoculations of infected amniotic fluid into rabbits. It contained antibodies to both V and S antigens. Haemagglutination-inhibition (HAI) titre 1/512; complement fixation (CF) titre 1/64.

(2) A/82/68. Influenza C antiserum produced by inoculations into rabbits of infected rhesus monkey kidney cell culture. HAI titre 1/1280.

(3) Anti-V serum. Influenza C anti-V serum was prepared in guinea-pigs by the method of Lief & Henle (1956, 1959). HAI titre 1/256; CF titre 1/64.

(4) Human convalescent serum. Influenza A/Hong Kong antiserum. CF titre 1/2048.

All antisera were treated with periodate according to the method of Nelson & Lewis (1958), then filtered through a modified Hemmings membrane filter. They were used at dilutions of 1/8 to 1/10.

Tissue cultures

The tissue cultures were maintained in equal parts of Eagle's MEM and 199 solutions containing penicillin (200 units/ml.) and neomycin (50 units/ml.).

Immunofluorescence technique

Primary monkey kidney cell sheets were prepared on cover-slips. The approximate number of cells per cover-slip was $3{\cdot}4 \times 10^5$. One drop of clarified amniotic fluid, containing about 2·5–5 egg infective particles of influenza C per cell, was placed on the cell sheet and left to adsorb at 30° C. for 1 hr. The inocula were then washed off with maintenance medium and the cover-slips incubated at 37° C. in a CO_2 incubator for various times. The cell sheets were fixed in acetone at −20° C. for 30 sec. and, after washing in phosphate buffered saline (PBS), antiserum was added and they were incubated at 37° C. for 30 min. in a moist atmosphere. After thorough washing with PBS, the preparations were counter-stained with conjugate for 30 min. at 37° C. in a damp atmosphere, then stained with Evans Blue, 1/2000 in water (w/v), and finally washed, dried and mounted. A series of preparations were also made with influenza A/Hong Kong virus.

Controls

In every test, one cover-slip was tested for haemadsorption to confirm that the cells were infected. The criteria of specificity of staining were: (*a*) immunological staining of infected but not of uninfected cells, (*b*) no staining when normal serum was substituted for antiserum, (*c*) no staining with influenza C antiserum of cells infected with Hong Kong virus.

Preparation of amniotic sections

Ten-day embryonated eggs were inoculated amniotically with 0·2 ml. of a 1/100 dilution of influenza C/Paris, having an HA titre of 1/64, and incubated at 37° C. for the required number of hours before harvest of the amnion. Uninfected

control amnions treated with SWAR or normal rabbit serum were also examined at every stage.

After washing in PBS the amnion was fixed by immersion in acetone at $-20°$ C. for 30 sec. After a further wash in PBS it was frozen and sections cut on a Slee microtome were collected on cover-slips. Thereafter the immunofluorescent staining procedure already described was followed.

RESULTS

Development of influenza C/Paris in amniotic membrane

A series of sections of infected amnion were examined at intervals of 0–45 hr. after inoculation. The sections were stained with A/82/68 rabbit antiserum and counter-stained with swine anti-rabbit gamma globulins. The sections showed virus development to be negligible at 3 hr., well established at 4 hr., and then progressively increasing until 45 hr., when the entire membrane was stained (Plate 1, figs. 1, 2). Controls throughout the series showed no fluorescence.

Development of influenza C/Paris in rhesus monkey kidney cells

A/4/69 rabbit antiserum (containing both S and V antibodies) and swine anti-rabbit gamma globulins produced no specific fluorescent staining 2–8 hr. after infection. Occasionally very fine, brightly fluorescing individual granules were observed on the surface of single cells, probably representing unadsorbed virus particles (Plate 2, fig. 1). No haemadsorption was detected in the tissue culture at this stage. At 10 hr., strong granular nuclear, nucleolar, and cytoplasmic fluorescence was observed. At no time during the experiments was a cell observed with only nuclear fluorescence (Plate 2, figs. 2, 3). A perinuclear halo was seen in some cells (Plate 2, fig. 3). At this stage the inoculated cell sheets showed slight haemadsorption. By 17 hr. the fluorescence was mainly cytoplasmic. Traces of remnant S antigen were seen in some nuclei giving them a stippled appearance (Plate 3, fig. 1). Perinuclear halos were still present in some cells, and haemadsorption had slightly increased.

At 21 hr. no nuclear fluorescence was seen and many more cells exhibited cytoplasmic fluorescence (Plate 3, fig. 2). Fluorescing multinucleate cells (Plate 4, fig. 1), cells with fibrous extensions (Plate 5, fig. 1) and also elongated cells were observed (Plate 4, fig. 2). A cytopathic effect began to develop in the cells (Plate 4, fig. 3), and haemadsorption showed a further increase. At 24–36 hr. nuclear fluorescence was again observed (Plate 5, fig. 2), presumably due to secondary infection since antiserum had not been added after virus had been allowed to infect the cells, and from 48 to 72 hr. the fluorescence was granular and cytoplasmic. Cells were beginning to detach from the cover-slips. Multiple patches of haemadsorption were now visible.

As with other influenza viruses, the nucleoli were prominent throughout the infection with influenza C virus, and fluoresced brightly during the nuclear stages of multiplication. Throughout the experiments only a small percentage of the cells were infected, usually in groups of 2 or 3.

Using anti-V serum on infected secondary monkey kidney cells and staining with SWAR only cytoplasmic fluorescence was observed from 10 to 12 hr. onwards.

Control with influenza virus A/Hong Kong

Primary monkey kidney cells inoculated with influenza A/Hong Kong were treated after 4, 6 and 8 hr. with convalescent antiserum and counter-stained by SWAR. At 4 hr. fluorescence was weak, but at 6 hr. strong nuclear fluorescence was present.

DISCUSSION

It has been shown by immunofluorescence of infected cells that soluble antigen of influenza A and B viruses is first synthesized in the nucleus and moves later to the cytoplasm (Watson & Coons, 1954; Lui, 1955; Breitenfeld & Schäfer, 1957; Franklin, 1958), whereas the antigen of myxovirus group 2 and paramyxoviruses has been detected only in the cytoplasm of cells (Wheelock & Tamm, 1959; Traver *et al.* 1960).

The influenza C/Paris virus investigated in the present paper conforms to the immunofluorescent pattern produced by influenza A and B viruses. Soluble antigen was first detected in the nucleus 10 hr. after infection. This is considerably later than the time of 3 hr. first reported for fowl plague virus (FPV) by Breitenfeld & Schäfer (1957) and 3 hr. reported for influenza A and B viruses by Watson & Coons (1954) and many subsequent workers. Our influenza A/Hong Kong control tests also took longer than 3 hr. (5–6 hr.) to develop strong nuclear immunofluorescence. The nucleoli exhibited strong fluorescence at 10 hr., as had been observed in FPV and influenza A and B infections. By 17–21 hr. only cytoplasmic fluorescence was found. Again, the time is much later than the 5–6 hr. for influenza A and B viruses reported by Breitenfeld & Schäfer (1957) and Watson & Coons (1954). No nuclear fluorescence was observed when specific anti-V serum was used. V antigen, also, first appeared in the cytoplasm 10 hr. after infection. At this time, too, weak haemadsorption confined to individual cells was noted. The appearance of considerable fluorescence in the amnion of infected embryo at 4 hr. approaches the time of development of antigen as reported by Breitenfeld & Schäfer (1957) and Watson & Coons (1954). Clearly, influenza C grows much more slowly in tissue culture than in eggs.

We wish to thank the technical staff at the Regional Virus Laboratory, East Birmingham Hospital, for their co-operation.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section of chick amnion 4 hr. after inoculation with influenza C/Paris. Outer cells of membrane (section between arrows) fluorescing. (Blue light, 15 sec.)

Fig. 2. Section of chick amnion 45 hr. after infection with influenza C/Paris. Entire membrane fluorescing. (Blue light, 10 sec.)

PLATES 2–5. Magnification × 400

PLATE 2

Fig. 1. Monkey kidney cells 6 hr. after infection with influenza C/Paris. Individual fluorescing granules. (Blue light 25 sec.)

Fig. 2. Single infected monkey kidney cell exhibiting bright nuclear and cytoplasmic fluorescence. (Blue light, 25 sec.)

Fig. 3. Central group of infected monkey kidney cells showing nuclear and cytoplasmic fluorescence. One cell (arrowed) has a perinuclear halo. (Blue light, 30 sec.)

PLATE 3

Fig. 1. Influenza C/Paris infected monkey kidney cell; 17 hr. Large central cell with granular fluorescing cytoplasm. The nucleolus is unstained and the nucleus contains granules of weakly staining remnant S antigen. (Blue light, 30 sec.)

Fig. 2. Influenza C/Paris infected monkey kidney cells at 21 hr. Five cells with bright cytoplasmic fluorescence; the nuclei are unstained. (Ultraviolet, 1 min.)

PLATE 4

Fig. 1. Influenza C/Paris infected monkey kidney cells; 36 hr. Large binucleate (nuclei arrowed) cell – bright cytoplasmic fluorescence, the nucleoli and nucleus are unstained. (Blue light, 15 sec.)

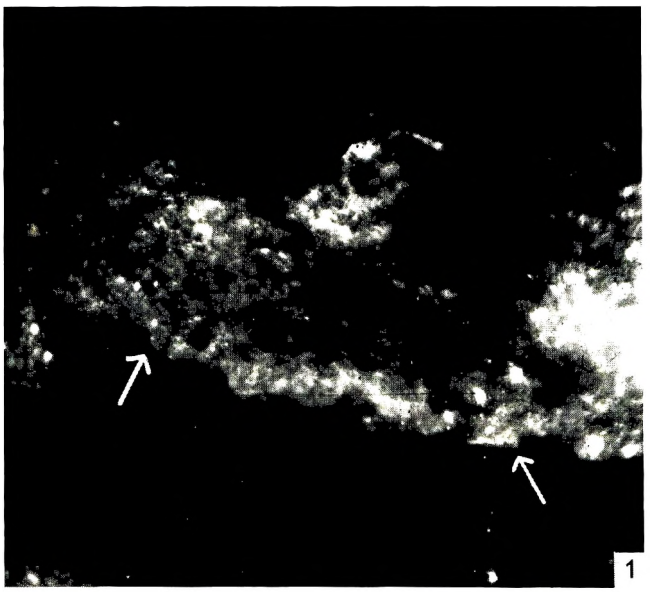
Fig. 2. Influenza C/Paris infected monkey kidney cells; 21 hr.; 1 central extended cell with cytoplasmic fluorescence only. (Ultraviolet, 45 sec.)

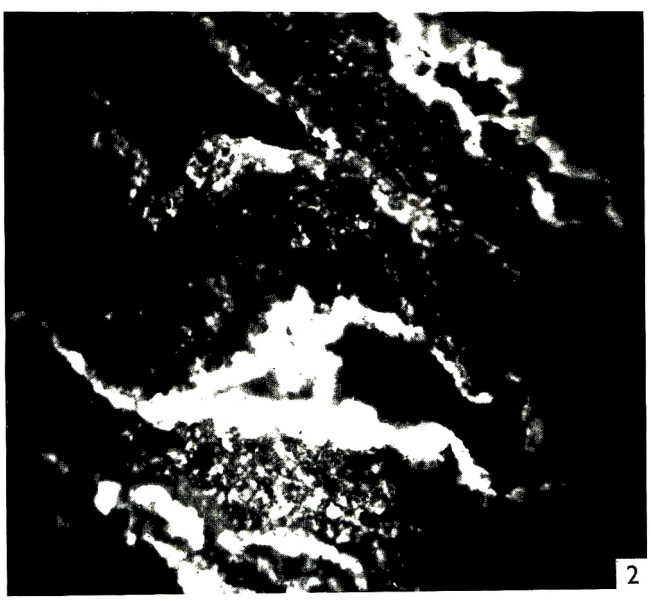
Fig. 3. Influenza C/Paris infected monkey kidney cells; 21 hr. Cytoplasmic fluorescence. Early cytopathic effect. Group of rounded cells – some with cytoplasmic fluorescence. (Ultraviolet, 80 sec.)

PLATE 5

Fig. 1. Influenza C/Paris infected monkey kidney cells; 21 hr. Large cell with fibrous extensions. Cytoplasmic fluorescence. (Blue light, 30 sec.)

Fig. 2. Influenza C/Paris infected monkey kidney cells; 36 hr. Strong nuclear and cytoplasmic fluorescence in two central cells. (Blue light, 15 sec.)





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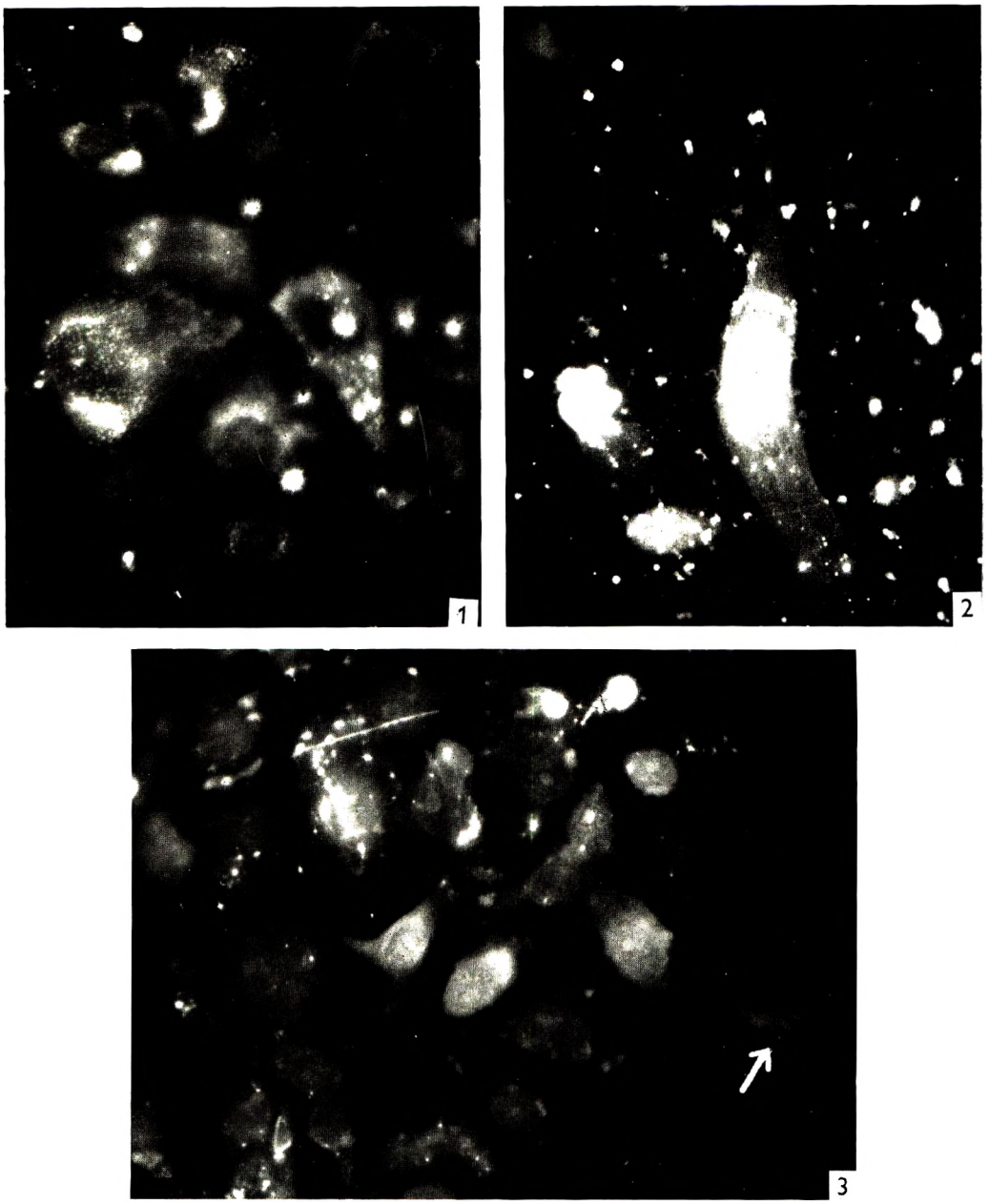
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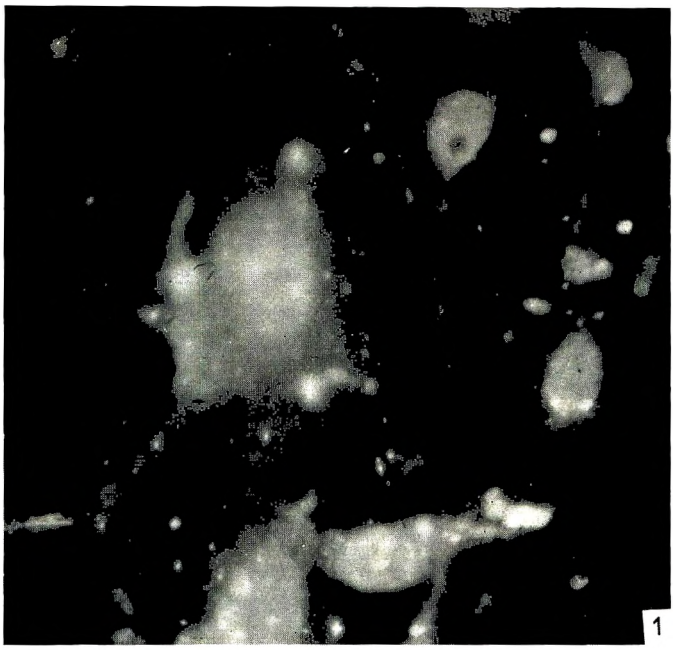
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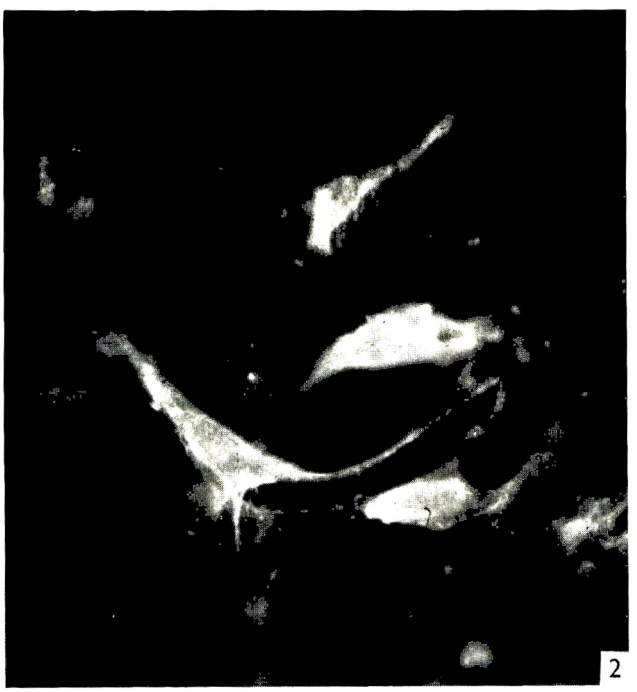
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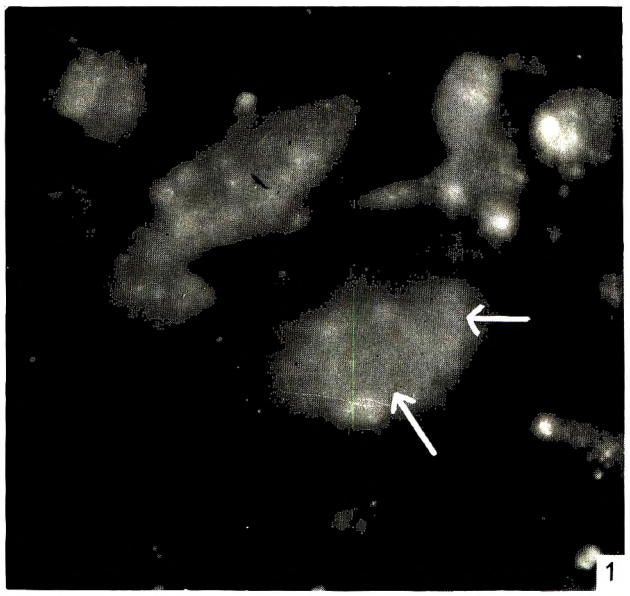
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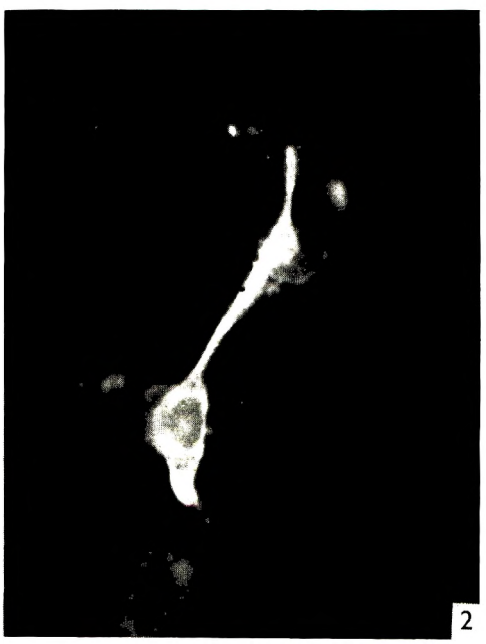
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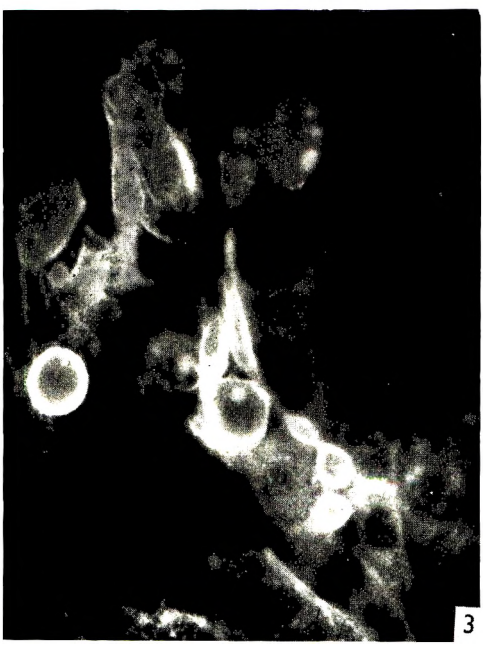
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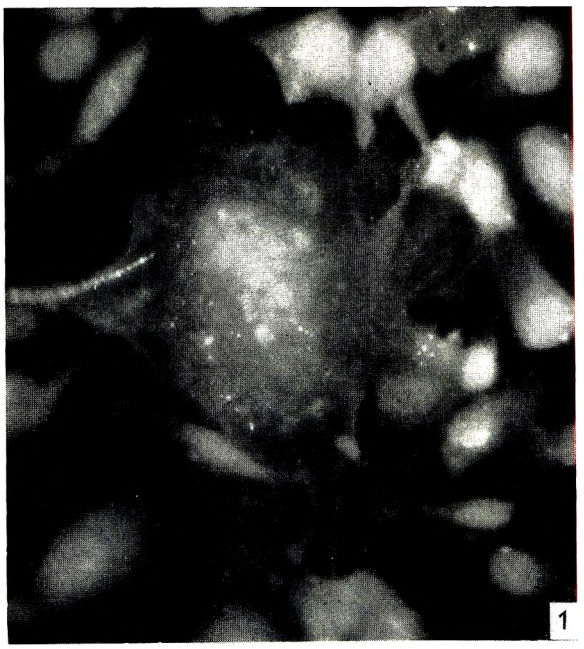
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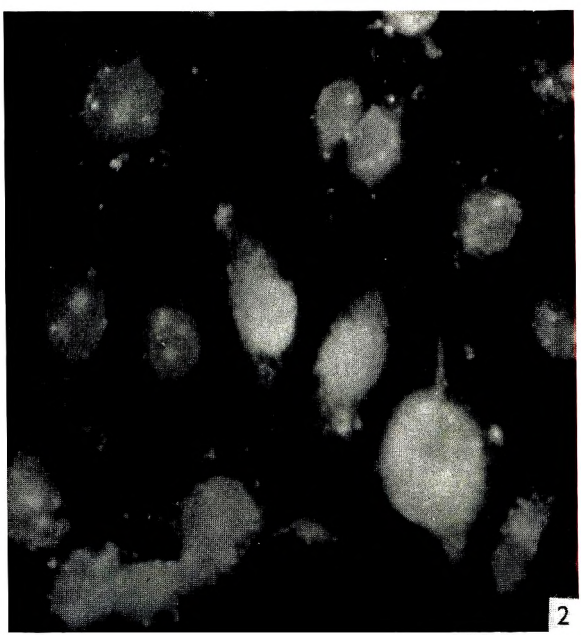
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The influence of pH of the culture medium on the sensitivity of *Mycoplasma gallisepticum* antigens for use in certain serological tests

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SUMMARY

Mycoplasma gallisepticum antigens were prepared from organisms cultured in broth medium with glucose. The influence of period of growth, pH of the medium and duration of incubation at low pH (5·0) on the sensitivity of these antigens was determined in certain tests. The most sensitive antigen for the serum plate test was harvested after no more than 8 hr. incubation at pH 5·0. Sensitivity in serum plate, haemagglutination and gel diffusion tests was impaired if organisms were incubated at pH 5·0 for long periods. Antigens prepared from buffered broth medium were found to be at least as sensitive as those from unbuffered medium for the haemagglutination and gel precipitation tests, but considerably less so for the serum plate test.

INTRODUCTION

Serological tests currently used for the diagnosis of *Mycoplasma gallisepticum* infection in poultry include the haemagglutination inhibition (HI), tube agglutination (TA), whole blood agglutination (WB) and serum plate agglutination (SP) tests.

None of these can be regarded as entirely satisfactory. The HI test yields a result within a few hours but normal chicken and turkey sera sometimes give false positive reactions (White, Wallace & Alberts, 1954; Roberts, Olesiuk & Van Roekel, 1967; Kulasegaram, 1967). Measures taken to avoid these non-specific HI titres may reduce the sensitivity of the test (Jordan & Kulasegaram, 1968*a*; Thornton, 1969). The TA test may be difficult to read (Jacobs, Jungherr, Luginbuhl & Gianforte, 1953), false positive reactions may occur (Roberts *et al.* 1967; Kulasegaram, 1967) and the results in one well-recognized method cannot be read for 24–48 hr (Mayeda & Lewis, 1969). The WB and SP tests have found wide application for the detection of *M. gallisepticum* antibodies because they are simple to perform and yield rapid results. However, such tests should be interpreted with caution since false positive reactions can occur due to a variety of factors (Thornton, 1969; Roberts, 1970).

Conflicting reports on the sensitivity of these tests (Jungherr, Luginbuhl, Tourtellotte & Burr, 1955; Leach & Blaxland, 1966; Jordan & Kulasegaram, 1968*a*; Thornton, 1969) suggest that they must be influenced both by the method of performing the tests and by the sensitivity of the antigen used. Considerable variations in sensitivity have been noted between *M. gallisepticum* agglutinating

antigens prepared in different laboratories (Barber, 1962; Halen & Schyns, 1969) and even between batches prepared in the same laboratory (Coller, Strout & Dunlop, 1955; Dunlop & Strout, 1956). Factors reported to influence the sensitivity of such antigens include the strain of the organism (Adler & DaMassa, 1965), the concentration of organisms (Halen & Schyns, 1969) and the diluents and preservatives used (Hromatka & Adler, 1969).

Other factors that may be of considerable importance are the type of medium and conditions of culture. Although various media have been used for the production of *M. gallisepticum* antigens, little has been reported on the effects of different media constituents or different conditions of growth on the subsequent sensitivity of the antigen. Adler & DaMassa (1968) found that *M. gallisepticum* antigens grown in broth with glucose, where the pH became acid, were less sensitive in the SP test than antigens grown in broth without glucose where the pH did not fall below 7·1. They concluded that the low pH had damaged the antigenic properties of the organism. In their experiments cultures were incubated for 7 or 8 days but the influence of the duration of incubation at low pH was not investigated.

Our own preliminary work had indicated that duration of incubation might influence the sensitivity of the resulting antigen, and we here report the results of further investigation into the effects of pH of the culture medium with particular reference to duration of incubation. We have also examined the sensitivity of antigen grown in buffered medium, as suggested by Adler & DaMassa in 1968.

MATERIALS AND METHODS

Mycoplasma gallisepticum *strains*

The following two strains were used.

A 514 obtained from Dr H. Chu, University of Cambridge. This strain had undergone numerous passages in artificial medium in this laboratory.

S 6 obtained from Dr D. H. Roberts, Central Veterinary Laboratory, Weybridge.

Culture media

Broth medium (BM). The broth used for routine maintenance of cultures and for growth of the antigens was that described by Bradbury & Jordan (1971) except that it contained 15% heat-inactivated swine serum instead of horse serum. The medium contained 0·1% glucose, and the initial pH was 7·5.

Buffered broth medium (BBM). BM was supplemented with 0·2 M phosphate buffer, a molarity necessary to maintain the pH of the culture above 6·7 for 3 days and allow good growth of the organism.

Agar medium (AM). This was similar to BM except that it contained 1% ion agar No. 2,* and phenol red was omitted.

Antigen preparation

BM, warmed to 37° C., was inoculated with one-tenth of its volume of a 24 hr. culture of *M. gallisepticum* in BM. The culture was incubated at 37° C. and in some experiments the number of viable organisms was determined just before

* Oxoid Ltd., London.

harvest. Organisms were harvested by centrifugation at 3000 g and the pH of the culture medium was measured. The organisms were washed three times in 0·02 M phosphate-buffered saline pH 7·0 (PBS), and in the earlier experiments the total yield of antigen was determined as described below. Antigens were tested for sterility and standardized to a concentration of 75 times an optical density of 40 on an E.E.L. nephelometer. This optical density was selected as being approximately equivalent to 15 times the turbidity of Brown's opacity scale number 4 (Jordan & Kulasegaram, 1968*a*).

Viable counts

These were performed by the method of Miles & Misra (1938). Serial tenfold dilutions of the culture were prepared in BM and five 0·02 ml. drops were immediately transferred from each dilution to AM, using one plate per dilution. Plates were incubated at 37° C. for at least 1 week before colonies were counted.

Total yield of antigen

This was measured in terms of turbidity on an E.E.L. nephelometer. Harvested antigens, after washing, were made up in PBS to 2·5 times their original concentration in BM. This was to bring the turbidity within the range of the nephelometer.

Chicken and turkey sera

These were from field outbreaks of *M. gallisepticum* infection. They were stored at 0–4° C. Serial dilutions of the sera for the SP test were prepared in PBS pH 7·0.

Rabbit sera

Serum was obtained from rabbits immunized with the A 514 strain of *M. gallisepticum*. The immunizing antigen was prepared from an 18 hr. broth culture, harvested and standardized as outlined above. The inoculation procedure was as described by Jordan & Kulasegaram (1968*b*) except that a multiple emulsion adjuvant (Herbert, 1967) was used instead of Freund's complete adjuvant.

SP tests

Tests were carried out at room temperature on clean grease-free microscope slides. A Marburg* micropipette was used to deliver 0·02 ml. of serum, or serum dilution and 0·02 ml. of the test antigen was similarly added. The slide was gently rocked for a standard reaction time of 2 min. for chicken sera and 3 min. for turkey sera (Jordan & Kulasegaram, 1968*a*). A known negative homologous serum was included in each series of tests. In each experiment antigens were compared by testing against four or five chicken sera.

HA tests

Serial twofold dilutions of the standardized antigen under test were made in PBS in Microtitre† disposable 'V' plates using microdiluters.† An equal volume

* Eppendorf, V. A. Howe, London.
† Flow Laboratories, Irvine, Scotland.

(0·05 ml.) of a 0·75% suspension of turkey red blood cells was added to each cup. A cell control containing 0·05 ml. PBS and 0·05 ml. red blood cell suspension was included in each test. The plate was shaken, allowed to stand at room temperature and results were read when the control cells had settled. The HA titre of the antigen was taken as the highest dilution of antigen giving complete haemagglutination.

Precipitin tests

Double diffusion in 1% agar gel was used to examine the precipitation reaction between the antigens and the serum from immunized rabbits, having first carried out tests to ensure that the reaction was specific, and not due to antigenic components of the medium (Jordan & Kulasegaram, 1968*b*). The gel was prepared from Noble agar (Difco) and contained 0·15 M sodium chloride. Results were photographed after incubation at 37° C. in a moist atmosphere for 48 hr.

RESULTS

Experiment 1

To study the effects of duration of incubation and consequent pH change of the medium on the sensitivity of *M. gallisepticum*, the A 514 strain was grown in BM and harvested after 1, 3, 5 and 7 days incubation. At the time of harvest a viable count was performed, the pH of the medium was measured and the total yield of

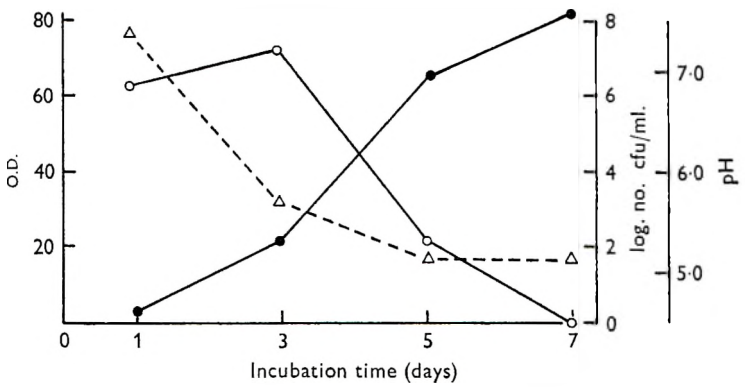

Fig. 1. The effect of incubation time on the viability, pH and antigen yield of an *M. gallisepticum* A514 culture (Expt. 1). O.D. = optical density; cfu = colony forming units. ●, O.D.; ○, cfu; △, pH.

antigen was determined (Fig. 1). The turbidity of the culture increased with incubation time; the number of viable organisms declined rapidly between the third and fifth day by which time the pH had fallen to 5·1. The pH was unchanged at day 7.

The antigen yield was insufficient for the SP and HA tests after only 1 day's incubation but the results for the other antigens (Table 1) showed a decline in both agglutinating sensitivity and HA activity with increasing incubation time.

Table 1. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after different periods of incubation (Expt.* 1)

			Incubation time (days)		
	Serum		3	5	7
SP	Chicken sera	1	80	20	10
		2	40	10	5
		3	10	5	5
		4	20	10	10
		5	20	20	10
	Turkey sera	6	40	20	20
		7	160	160	80
		8	20	20	20
		9	10	10	5
		10	10	10	10
HA			32	8	4

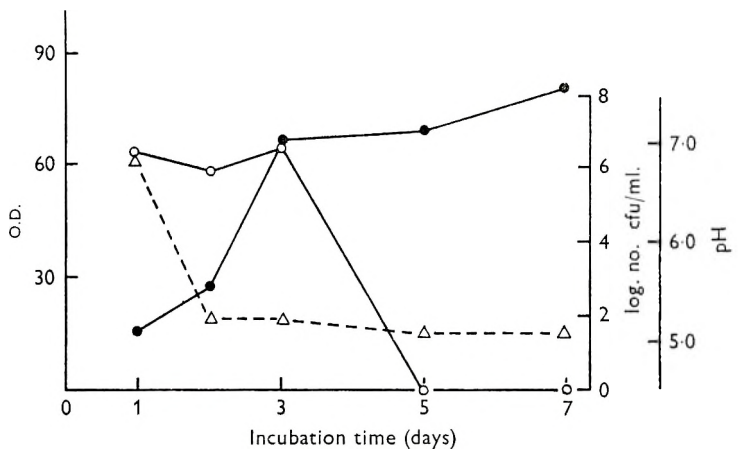

Fig. 2. The effect of incubation time on the viability, pH and antigen yield of an *M. gallisepticum* A 514 culture (Expt. 2). O.D. = optical density; cfu = colony forming units. ●, O.D.; ○, cfu; △, pH.

Experiment 2

This experiment was similar to Expt. 1 but antigen was also harvested after 2 days incubation. The pH fell more rapidly than in Expt. 1 and reached 5·2 by the second day (Fig. 2). As in the previous experiment, the turbidity of the culture increased steadily with incubation time even after day 5 when no viable organisms could be detected. In the SP and HA tests maximum sensitivity was shown by the antigen harvested after 2 days incubation (Table 2) and a decline in sensitivity with increased incubation time was again noted. Plate 1 A shows the result of the gel diffusion test. The antigens harvested after 1 and 2 days showed a strong reaction but this decreased both in intensity and number of precipitin lines with increasing duration of incubation.

Table 2. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after different periods of incubation* (*Expt.* 2)

			Incubation time (days)				
		Serum	1	2	3	5	7
SP	Chicken sera	11	5	20	10	10	5
		12	5	5	5	5	1
		13	20	80	40	20	10
		14	N	1	1	1	1
		15	1	10	5	5	1
	Turkey sera	16	160	640	160	80	80
		17	40	160	80	20	20
		18	20	40	40	20	20
		19	40	40	40	20	20
		20	40	160	40	40	40
HA			64	512	16	8	4

N = negative.

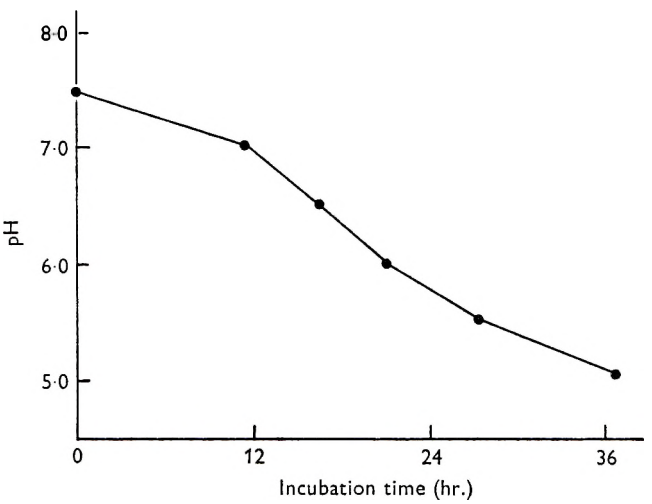

Fig. 3. The effect of incubation time on the pH of an *M. gallisepticum* A 514 culture (Expt. 3).

Experiment 3

Although Expts. 1 and 2 indicated that prolonged incubation at low pH diminished the sensitivity of the antigen in all three tests, it was not possible to determine if there was a critical pH below which sensitivity was impaired. Therefore, in this experiment organisms were harvested as soon as the medium had fallen to pH values 7·0, 6·5, 6·0, 5·5, and 5·0. A recording meter* was used to monitor pH. The incubation times at which these pH values were reached are shown in Fig. 3.

The SP and HA titres of the antigens showed no definite pattern (Table 3) but the total incubation time of this experiment was relatively short.

* Analytical Measurements Ltd., Richmond.

There was little difference in the precipitin reactions of the antigens (Plate 1 B). All showed a wide, intense precipitation band approximately midway between antigen and serum but with antigen harvested at pH 5·5 or 5·0, only one other line was visible near the outer wells while several were visible with the other antigens.

Table 3. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting at different pH values* (*Expt.* 3)

			pH				
		Serum	7·0	6·5	6·0	5·5	5·0
SP	Chicken sera	21	10	10	10	10	10
		22	20	10	10	20	20
		23	1	N	N	N	1
		24	1	N	N	N	1
		25	5	N	N	N	1
	Turkey sera	26	10	10	10	20	20
		27	10	5	5	5	5
		28	20	20	20	20	40
		29	1	1	1	1	1
		30	1	1	1	1	1
HA			128	256	128	128	128

N = negative.

Table 4. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after varying incubation times at pH* 5·0 (*Expt.* 4)

			Total incubation time (hr.)					
			46	54	62	70	78	86
			Incubation time at pH 5·0 (hr.)					
		Serum	8	16	24	32	40	48
SP	Chicken sera	31	5	NT	5	5	5	5
		32	40	NT	20	10	10	10
		21	5	NT	5	5	5	5
		22	40	NT	40	10	10	10
	Turkey sera	26	160	NT	80	80	80	80
		28	160	NT	40	40	40	40
		33	320	NT	80	80	80	80
		34	80	NT	40	40	40	40
HA			64	64	8	8	4	4

NT = not tested because antigen became contaminated.

Experiment 4

To investigate the effects of continued incubation at low pH, a culture was grown until the pH reached 5·0, a period of 38 hr. being required. Antigens were harvested after incubation for 8, 16, 24, 32, 40 and 48 hr. at pH 5·0.

A decline in SP sensitivity of the antigens was observed between 8 and 32 hr.

(Table 4). The HA titre of the antigens fell rapidly between 16 and 24 hr. The decline in SP titre appeared to be more gradual, although the antigen harvested after 16 hr. incubation at pH 5·0 was not included in these tests owing to bacterial contamination. (The HA and gel diffusion tests were conducted before the antigen became contaminated.) Examination of the antigens by agar gel diffusion also showed fewer precipitin lines with prolonged incubation of the culture (Plate 2C).

Experiment 5

To extend the previous experiment, cultures were grown to pH 5·0 (38 hr.) and organisms harvested after 48, 56, 64 and 72 hr. incubation at that pH. There was little difference in the SP or the HA titres of the four antigens (Table 5). Two of the chicken sera showed a slight fall in titre with the 64 and 72 hr. antigens but all other SP titres were constant.

Table 5. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared by harvesting after varying incubation times at pH* 5·0 (*Expt.* 5)

			Total incubation time (hr.)			
			86	94	102	110
			Incubation time at pH 5·0 (hr.)			
		Serum	48	56	64	72
SP	Chicken sera	31	5	5	1	1
		32	10	10	5	5
		21	5	5	5	5
		22	10	10	10	10
	Turkey sera	26	20	20	20	20
		28	20	20	20	20
		33	40	40	40	40
		34	20	20	20	20
HA			16	8	8	8

On examination by gel diffusion (Plate 2E) all the antigens showed a diffuse precipitin line together with a less distinct line nearer the antigen wells. Both lines were more clearly defined with 48 hr. antigen than with the other three.

Experiment 6

In order to determine whether prolonged incubation of culture at pH 5·0 would affect the sensitivity of the antigen if a different strain of organism were used, Experiment 4 was repeated with the S6 strain. The pH of the culture had fallen to 5·0 by 30 hr. and antigens were harvested after 8, 16, 24, 32, 40 and 48 hr. incubation at that pH. Since the pH of the medium fell more rapidly with this strain of *M. gallisepticum* than A 514, the total incubation times were shorter.

The SP and HA titres of the S6 antigens fell with increasing incubation time at pH 5·0, the greatest loss in sensitivity occurring between 24 and 32 hr. (Table 6).

On gel precipitation these antigens gave at least four lines although those with the 48 hr. antigens were less intense than the others (Plate 2D).

Table 6. *Reciprocal SP and HA titres of* M. gallisepticum *S6 antigens prepared by harvesting after varying incubation times at pH* 5·0 (*Expt.* 6)

			Total incubation time (hr.)					
			38	46	54	62	70	78
			Incubation time at pH 5·0 (hr.)					
		Serum	8	16	24	32	40	48
SP	Chicken sera	31	40	40	40	1	1	1
		32	40	40	40	10	10	10
		21	10	10	10	5	5	5
		22	40	40	40	20	20	20
	Turkey sera	35	40	40	40	10	10	10
		36	80	80	80	80	40	40
		37	80	80	80	40	40	40
		38	10	5	5	5	5	5
HA			16	16	16	8	8	8

Experiment 7

Adler & DaMassa (1968) associated loss of *M. gallisepticum* S6 antigen sensitivity with low pH of the medium because they found that a more sensitive SP antigen was obtained from medium without glucose, in which a fall in pH did not occur.

In an attempt to confirm these findings using A514, the organism was passaged ten times in broth without glucose, but even after the tenth passage the pH of the medium fell to 5·0–5·1. Similar results were obtained in broth containing horse serum in place of swine serum. It was concluded therefore that the sera contained fermentable carbohydrates and that the work of Adler & DaMassa (1968) could not be repeated.

Experiment 8

This experiment was designed to compare the sensitivity of antigens prepared from buffered broth medium (BBM) with those prepared from broth medium (BM). *M. gallisepticum* A514 was cultured in BBM and in BM. The experiment was conducted four times in all, twice with an incubation time of 48 hr. (i and ii) and twice with an incubation time of 72 hr. (iii and iv). The pH values of the media at the time of harvest are shown in Table 7.

Antigens prepared from BBM were more easily suspended in PBS and gave a finer suspension than those from BM. The SP and HA titres of 48 hr. antigens from (i) and (ii) are shown in Table 8 and those of 72 hr. antigens from (iii) and (iv) in Table 9. All the antigens from BBM were less sensitive in the SP test than those from unbuffered broth. There was no significant difference in HA titres of the antigens in (i) and (ii) but in (iii) and (iv) antigens prepared from BBM showed considerably higher titres than those from BM.

The antigens from (iv) were examined by gel diffusion (Plate 1A) and that from the BBM (3*b*) showed 2 intense precipitin bands and at least one other band while the antigen from BM (3) showed only two faint bands.

Table 7. *pH of the media at harvest* (*Expt.* 8)

Expt.	(i)		(ii)		(iii)		(iv)	
Incubation time (hr.)	48		48		72		72	
Medium	BBM	BM	BBM	BM	BBM	BM	BBM	BM
pH	6·8	5·1	6·9	5·0	6·7	5·7	6·7	5·1

BBM = buffered broth medium. BM = broth medium.

Table 8. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared from cultures grown in buffered broth medium* (*BBM*) *and broth medium* (*BM*) *for* 48 *hr.* (*Expt.* 8)

		i			ii	
	Serum	BBM	BM	Serum	BBM	BM
SP, Chicken sera	39	5	20	49	5	10
	40	1	5	50	N	1
	41	20	160	51	N	1
	42	N	5	52	N	1
	43	1	10	53	N	1
SP, Turkey sera	44	40	160	54	40	160
	45	20	80	55	20	80
	46	10	10	56	10	40
	47	20	20	57	20	40
	48	20	40	58	20	40
HA		32	32		32	16

N = negative.

Table 9. *Reciprocal SP and HA titres of* M. gallisepticum *A* 514 *antigens prepared from cultures grown in buffered broth medium* (*BBM*) *and broth medium* (*BM*) *for* 72 *hr.* (*Expt.* 8)

		iii			iv	
	Serum	BBM	BM	Serum	BBM	BM
SP, Chicken sera	1	5	80	11	5	10
	2	1	40	12	1	5
	3	1	10	13	10	40
	4	1	20	14	N	1
	5	1	20	15	N	5
SP, Turkey sera	6	10	40	16	40	160
	7	160	160	17	20	80
	8	10	20	18	10	40
	9	5	10	19	10	40
	10	5	10	20	20	40
HA		128	32		512	16

N = negative.

DISCUSSION

The results of Expts. 1 and 2, which clearly show that loss of *M. gallisepticum* antigen sensitivity is associated with low pH of the culture medium, confirm the findings of Adler & DaMassa (1968).

In Expt. 1 the sensitivity of the antigens in SP and HA tests declined with increasing incubation time. However, in addition to a fall in the pH of the medium there was a decrease in the number of viable organisms and it was thought that both factors might have contributed to the decrease in antigen sensitivity.

In a similar experiment (2) a decline in antigen sensitivity with increasing incubation time was again noted in SP, HA and in gel precipitation tests. This decline was particularly evident between 2 and 3 days of incubation, when the number of viable organisms was fairly stable suggesting that death of the organism was not responsible for lowered sensitivity. A more likely cause was the acid pH of the medium. This had fallen rapidly and to a minimum by day 2 and, therefore, by day 3 the organisms had been at low pH for at least 24 hr. The inhibitory effects of low pH have also been noted by Pollock, Somerson & Senterfit (1969) in connexion with *M. pneumoniae* antigen sensitivity. They found that antigens harvested from medium which had become acid lost their ability to induce antibody production when inoculated into animals, and to act as antigens in immunodiffusion tests.

The next experiments investigated the effects of pH in more detail. In Expt. 3 organisms harvested as soon as the pH of the medium had fallen to specific values between 7·0 and 5·0 showed no obvious loss in sensitivity, suggesting that reduced sensitivity was probably associated with continued incubation of cultures after the pH had fallen to 5. Expts. 4 and 5 studied the effects of continued incubation on the antigenic properties of the organism. Cultures harvested at 8-hourly intervals after the pH of the medium had reached 5·0–5·1 exhibited a loss in sensitivity with increasing time. Clearly, therefore, *M. gallisepticum* organisms intended for use as antigens should not remain in medium of low pH for more than a few hours.

It seems likely that there may be an optimal time at which to harvest *M. gallisepticum* for SP antigens. Evidence for this view is provided by Expts. 2, 3 and 4. In Expt. 2 the antigen prepared after 1 day's incubation was not as sensitive in SP tests as that from 2 days incubation, although the pH was low by day 2. In Expts. 3 and 4 the titres of the two turkey sera (Nos. 26 and 28) that were common to both experiments were four- to eightfold higher with the antigen harvested after 8 hr. incubation at pH 5·0 than that from organisms harvested as soon as the pH had reached 5·0.

Thus it appears that, while incubation of A 514 antigens at low pH for more than 8 hr. will damage antigenic properties, a shorter period of incubation at low pH may actually enhance them. There is no ready explanation for this. It is possible that the first effect of low pH on the antigen suspension is to reduce its stability so that it is more readily agglutinated by addition of antibody. Another influencing factor might be the morphology of the organisms. This is known to vary with pH

(Freundt, 1969; Anderson, 1969) and might also influence agglutination properties. An electron microscope study of morphology in relation to antigenic sensitivity might prove of value in this context.

Loss in sensitivity with increasing incubation time at pH 5·0 is clearly not confined to the A 514 strain of *M. gallisepticum* since the S6 strain of the organism exhibited the same property.

The experiment (7) designed to repeat the conditions for antigen production with little fall in pH, by the omission of glucose (Adler & DaMassa, 1968) was unsatisfactory, since omission of glucose from the medium failed to prevent such a fall. Therefore, organisms were cultured in BBM so that the pH of the medium did not fall below 6·7 on incubation for up to 72 hr. Antigens prepared from this medium proved to be considerably less sensitive in SP tests than those prepared from unbuffered medium although HA titres were the same or higher and the number and intensity of gel precipitation lines were greater. An explanation for the SP and HA results may be that antigens prepared from BBM, being in a fine suspension, might have required a larger number of antibody molecules to produce a visible SP agglutination than antigens from BM which were already partially clumped. On the other hand, a fine suspension of antigen would favour HA reactions since there would probably be more HA sites available. The improved gel precipitation reaction is not easily explained but it is interesting that Pollack *et al.* (1969) could prevent the loss of *M. pneumoniae* antigen reactivity in gel tests caused by acidity of the medium by growing the organism in buffered broth.

The adverse effect of low pH with increasing duration of incubation remains unexplained but it is almost certainly associated with some irreversible alteration of the cell membrane. Pollack *et al.* (1969) concluded that, while low pH was an important factor in influencing *M. pneumoniae* sensitivity, the effect was probably not solely a hydrogen ion effect because incubation of organisms in buffers of low pH did not affect their reactivity in gel diffusion tests. Loss in reactivity may, as these authors suggest, be the result of enzymic action, but results in this laboratory (Bradbury & Jordan, in preparation) suggest that proteins from the culture medium may also play some role in altering antigen sensitivity. These proteins become firmly attached to the surface of the organism with increasing incubation time in acid medium, and could, in theory, affect antigenicity by masking antigenic sites. Results from Expts. 1 and 2 suggest that the antigens become increasingly contaminated with non-specific material since turbidity increased steadily even when there were no longer any detectable viable organisms. A similar observation was made by Eng (1969) in the production of *M. pneumoniae* antigen.

On the basis of our experimental findings certain suggestions can be made for the production of sensitive *Mycoplasma gallisepticum* antigens.

1. For serum plate tests, organisms should be grown in unbuffered medium and harvested after no more than 8 hr. at low pH.

2. For HA and gel precipitation tests, antigens may be prepared as above, or, more conveniently, organisms may be grown in buffered medium where the time of harvest is not critical.

3. Although our observations have not included other serological tests it is

possible that antigen sensitivity may be similarly affected and therefore merits further investigation.

We wish to thank Mrs C. A. Barratt for technical assistance and the Agricultural Research Council for financial support.

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EXPLANATION OF PLATES

PLATE 1

A. Gel precipitation reactions of *M. gallisepticum* A 514 antigens prepared by harvesting after different periods of incubation (Expt. 2). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 1, 2, 3, 5, 7 = number of days incubation. 3*b* = antigen prepared from buffered broth (Expt. 8).

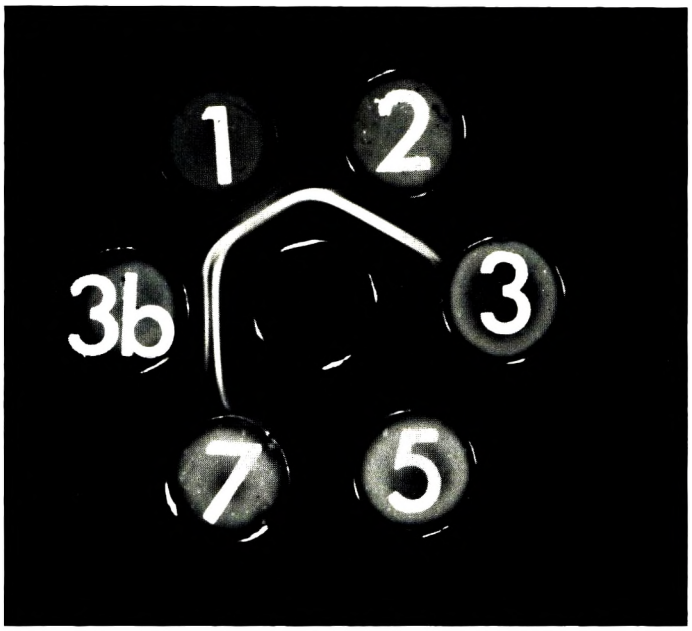
B. Gel precipitation reactions of *M. gallisepticum* A 514 antigens prepared by harvesting at different pH values (Expt. 3). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 7·0, 6·5, 6·0, 5·5, 5·0 = pH at which organisms were harvested.

PLATE 2

C. Gel precipitation reactions of *M. gallisepticum* A 514 antigens prepared by harvesting after varying incubation times at pH 5·0 (Expt. 4). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 8, 16, 24, 32, 40, 48 = number of hours incubation at pH 5·0.

D. Gel precipitation reactions of *M. gallisepticum* S 6 antigens prepared by harvesting after varying incubation times at pH 5·0 (Expt. 6). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 8, 16, 24, 32, 40, 48 = number of hours incubation at pH 5·0.

E. Gel precipitation reactions of *M. gallisepticum* A 514 antigens prepared by harvesting after varying incubation times at pH 5·0 (Expt. 5). The central well contained rabbit antiserum to *M. gallisepticum* and the outer wells contained the antigens. 48, 56, 64, 72 = number of hours incubation at pH 5·0.



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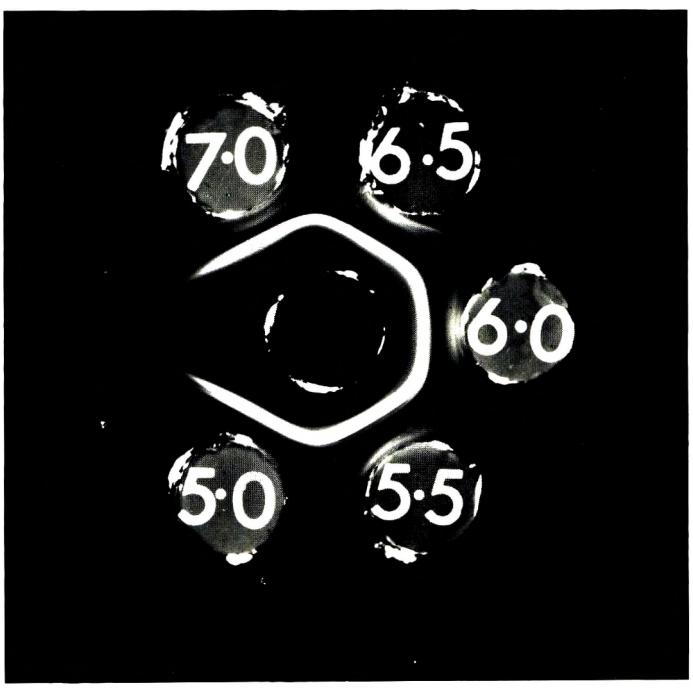

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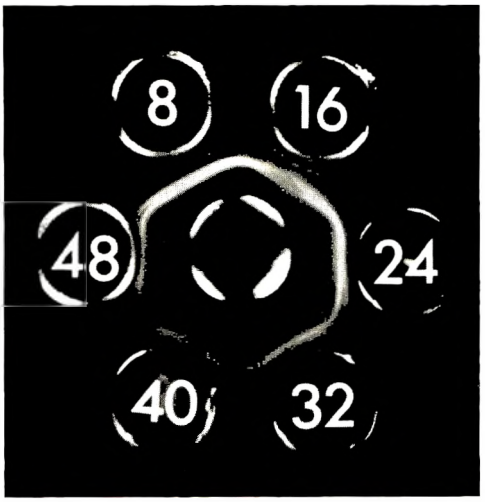

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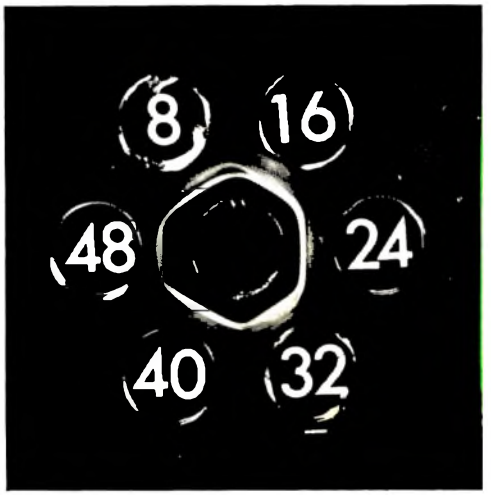

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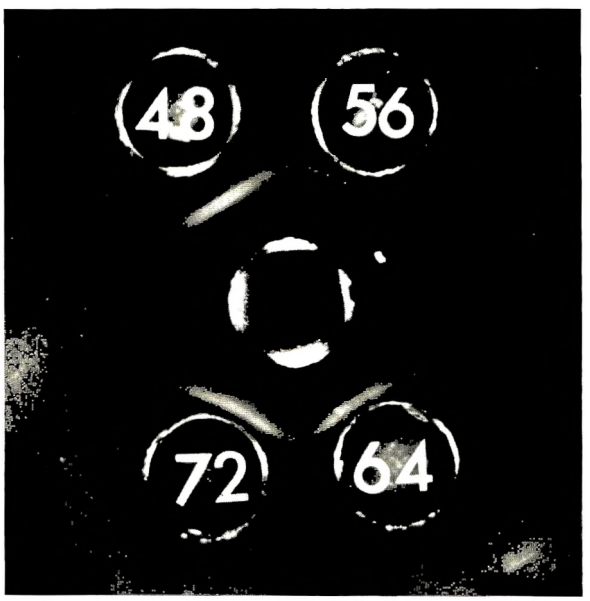

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An indoor system for the study of biological aerosols in open air conditions

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SUMMARY

An indoor system designed for the study of survival of airborne micro-organisms in closed conditions has been successfully modified to allow the effect of open air to be measured. It was found that the unidentified open-air factors which are toxic for many species of microbes and rapidly lost when enclosed in conventional laboratory apparatus could be retained in the system by continuous ventilation at an adequate rate. The rate required allowed examination of *Escherichia coli* in aerosols generated from small amounts of material because of the short periods of ventilation required for appreciable viable decay to occur.

The validity of the system was tested by comparing the survival of *E. coli* in true aerosols with its survival when the droplets were held on microthread. An investigation of the role of relative humidity in open-air toxicity was included.

INTRODUCTION

The study of influence of open air on the viability of micro-organisms was facilitated by the development of the microthread technique for exposing them in a simulated airborne state (May & Druett, 1968). Unidentified pollutants in rural air have been described which are adverse to microbial survival and further characterized by their rapid removal from air when it is enclosed (Druett & May, 1968). Such ephemeral air pollutants cannot be demonstrated in conventional laboratory apparatus and the obvious hazards prevent a study of their effect on survival of aerosols of pathogenic micro-organisms in the field. A new technique is clearly required. The present investigation was made to determine whether an enclosed system could be ventilated in a way that would preserve these air pollutants for a sufficient time for their bactericidal effect to be manifest. If this could be achieved the study of survival and respiratory infectivity characteristics in open air of aerosols of pathogenic micro-organisms would be considerably enhanced.

MATERIALS AND METHODS

A system normally used for examination of aerosols held under hermetically sealed conditions was used. It consists essentially of a 22 ft. diameter mild-steel sphere (vol. $1{\cdot}67 \times 10^5$ l.) with a by-pass tube (side-arm) from which aerosol samples can be withdrawn and animals exposed (Fig. 1). An orifice of 1 ft. in

diameter in the top of the sphere is exposed to allow access of spray devices for aerosol dissemination. Air mixing inside the sphere is achieved by three fans situated near the base and when required air from the sphere is drawn through the side-arm by a fan at one end of the arm. The sphere is housed in a brick chamber with a concrete floor and the side-arm passes through one wall to an adjoining chamber of similar construction. The sphere chamber has access doors at the top and bottom and four extract fans in its roof to assist natural ventilation. These fans give a theoretical air change rate in the sphere chamber of about 20 air changes/hr.

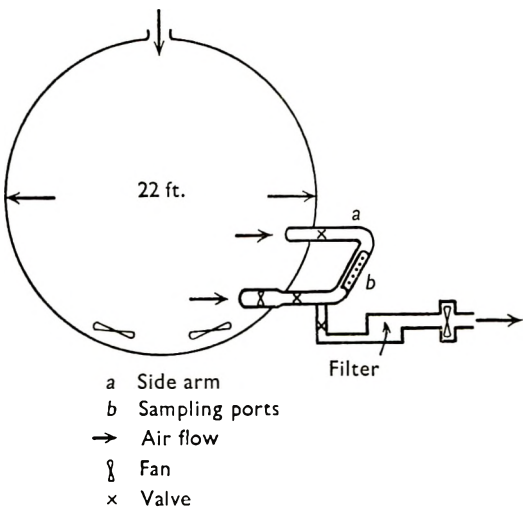

Fig. 1. Sphere system.

Modifications were made to this system to enable the sphere itself to be ventilated at rates of up to a maximum of 28 air changes/hr. Air is removed from the sphere via a T junction added to the side-arm, using a constant-speed extractor fan behind a triple bank of absolute filters, and replaced by air from the ventilated sphere chamber via the orifice in the top of the sphere. Ventilation rate was controlled by varying the restriction of the sphere air intake with knife-edged orifices of various diameters. Flow rate measurements were made with a Velometer (AEI Ltd) at the air intake. During these measurements a 1 ft. diameter tube 6 ft. long was attached to the intake to provide a laminar air flow.

E. coli MRE 162 (EC) was used to indicate, according to its viable decay rate, the toxicity of the air to which it was exposed. Spores of *B. subtilis* var. *niger* (BG) which are unaffected by the atmosphere were mixed with the EC to measure physical decay. Viable counts were made and EC viability assessed as described by May & Druett (1968). Aerosol samples were collected in multistage samplers (May, 1966) containing the collecting fluid described (May & Druett, 1968). In ventilated aerosol tests samples could be collected in still air from the side-arm by using a valve to by-pass the sampling ports without interruption of aerosol ventilation in the sphere. This was essential when efficient collection of large aerosol particles was required.

For aerosol tests about 3×10^{11} EC plus 1×10^{11} BG were sprayed directly into the centre of the sphere. When ventilated, loss of aerosol concentration due to physical removal proceeded at an exponential rate – about 90% loss in concentration every 10–15 min. Air samples of 500 l. were therefore required after 1 hr. ventilation in order to collect an adequate number of cells for assay. Two types of spray were used for dissemination of aerosols: a Collison spray with 18 jets which discharged about 1 ml. of fluid/min. when operated at 26 lb./in.^{2} air pressure and a two-fluid atomizer of the scent-spray principle operated at 80–100 lb./in.^{2} to discharge about 1 ml. fluid/sec. The former was used for production of small particulate aerosols of predominantly 1–2 μm. diameter and the latter for production of aerosols of much wider range of particle size.

In tests using the ventilated system, ventilation was started about 30 min. before exposure of micro-organisms. This established conditions of temperature and relative humidity very similar to those outside the building. Ventilation was continued without interruption until the end of the test. The high rates of ventilation used ensured that if a slight temperature differential existed between inside and outside the building the relative humidity was not affected by more than a few per cent.

Microthreads were loaded with particles containing EC/BG as described by May & Druett (1968). When they were exposed to open air during daylight hours they were housed in a roundabout (Druett & May, 1969) which shaded them from direct sunlight without preventing free access of air to them. When exposed to air in the sphere the frames were held in a wooden base by their handles, lowered to within a few feet of the bottom of the sphere and withdrawn at appropriate intervals during ventilation. Alternatively, they were inserted into exposure ports in the sphere side-arm. To avoid the adverse effect of air velocity on EC held on microthreads care was taken when possible to expose them in positions where the velocity would not be expected to influence survival (May & Druett, 1968). When this could not be done the microthreads were exposed simultaneously to similar air velocities in various parts of the system.

RESULTS

Introduction of toxic air to the system

All tests unless stated otherwise were made within the 70–95% range of relative humidity and between 3° and 20° C. Under such conditions the EC did not lose viability significantly during the exposure period in the non-ventilated system. The effect of ventilation was measured by comparing EC survival on microthreads held simultaneously inside the sphere and outside in open air. The results obtained at various ventilation rates indicated that (*a*) with 8 air changes/hr. no toxic air factors were present in the sphere, (*b*) with 9 air changes a proportion were present, and (*c*) with 12–20 air changes all toxic factors were apparently present since with these rates there was no difference between viable decay rate of EC inside and outside the sphere. Typical results are shown in Figs. 2 and 3.

The varying degree of toxicity of air from day to day was apparently associated

with the amount or nature of these air pollutants in the local air stream prevailing at the time of the test. From the results obtained it was concluded that a ventilation rate of over 12 air changes/hr. in the sphere was sufficient to maintain these pollutants at the same effective level as that found in open air in the locality.

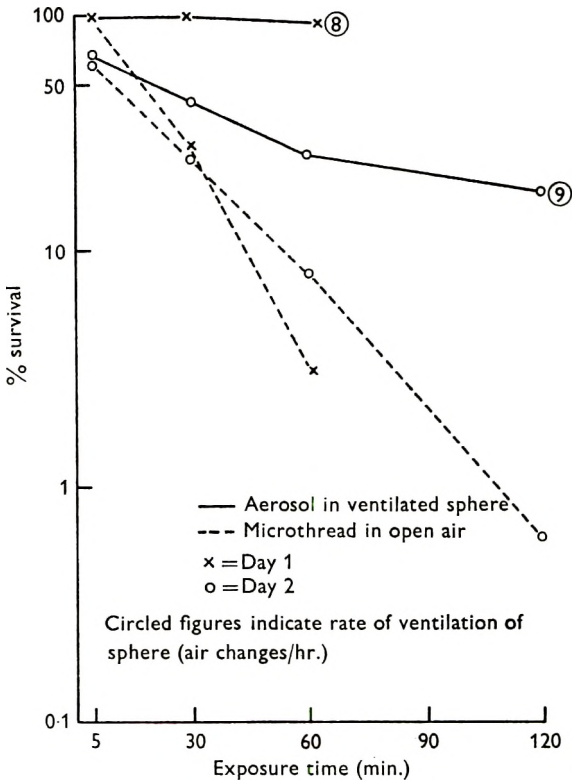

Fig. 2. *E. coli* survival on microthread in open air and simultaneously in aerosols in sphere ventilated at 8 and 9 air changes/hr.

Loss of toxic factors on enclosure

Loss of toxic factors in the air inside the sphere was very rapid when ventilation ceased. No further loss of EC viability in aerosols was observed when measured during subsequent periods of at least 20 min. (Table 1).

Ventilated aerosols and microthread exposure

Survival of EC in airborne particles $\leqslant 5\ \mu$m. diameter (i.e. those collected in the bottom stage of the multistage sampler) in the adequately ventilated sphere was compared with that in similar sized particles held simultaneously on microthread in open air. The results (Fig. 4 and Table 2) showed a slight difference – just significant ($P = 4{\cdot}8\%$) – between survival at 1 hr. in true aerosol and on microthread. The EC apparently survived slightly better on microthread than in the true airborne state.

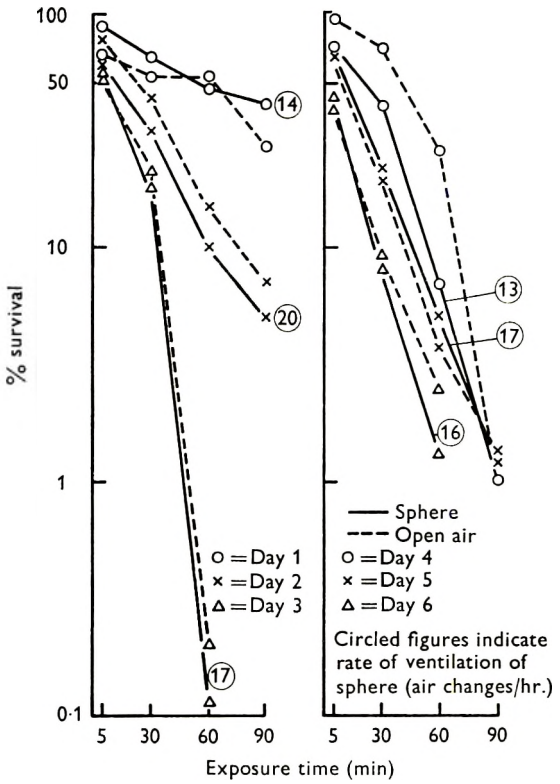

Fig. 3. *E. coli* survival on microthread in open air and simultaneously in sphere ventilated at rates between 12 and 20 air changes/hr.

Table 1. Escherichia coli *survival* (%) *in aerosols of* ⩽ 5 *μ particles: effect of ventilation for* 45 *min. followed by closed conditions* 45–65 *min.*

Aerosol age (min.)	Ventilated			Non-ventilated		
	5	30	45	55	60	65
Test (day 1)	51	49	6	7	7	7
Test (day 2)	63	37	2	2	1	3

Particle size response to toxic air

The results of exposing EC in aerosols and on microthread to solar radiation indicated that survival was poorer in small particles than in large ones (May & Druett, 1968). The practical difficulties limited those aerosol tests to airborne holding times of about 3·5 min. These are overcome by using the present system in which aerosols can be held for long periods. Tests for the effect of open air on true aerosols in the absence of sunlight could thus be made in the sphere at any time of day. The purpose was to determine whether the open air toxic factors exhibited a particle size effect on EC survival in the absence of sunlight.

Aerosols of wide range of particle size were ventilated and samples removed at intervals during their 1 hr. period of ageing. The three particle-size fractions

(< 3, 3–6 and > 6 μm. diameter) obtained with the multistage sampler were assessed for EC viability and decay curves plotted. The results showed that the effect of toxic air on EC survival was almost invariably associated with a particle-size effect, viable decay proceeding more rapidly as particle size diminished. The difference between the viable decay rates of the three particle-size fractions was

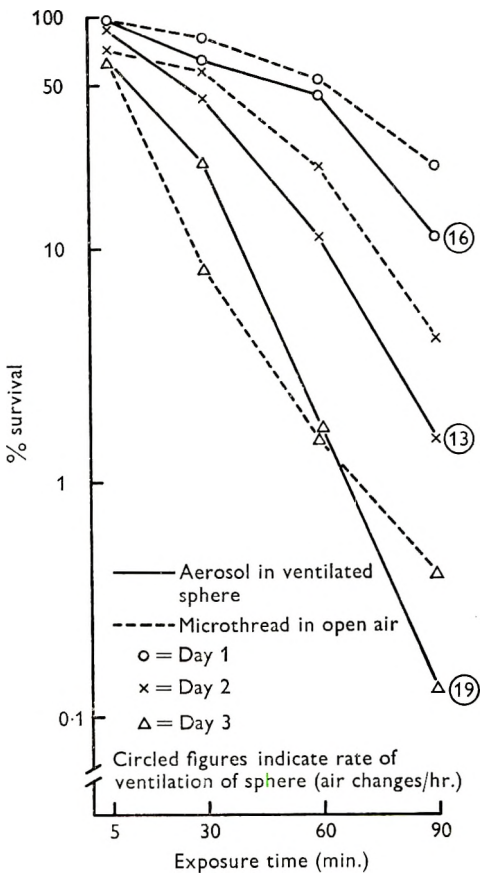

Fig. 4. *E. coli* survival on microthread in open air and simultaneously in aerosols in ventilated sphere.

not constant from one test to another even when relative humidity and temperature conditions were similar. Some examples of the types of response obtained are shown in Fig. 5. The results confirm the conclusions reached by Druett & May (1968) from their work with microthread, that the nature and concentration of the unstable germicidal pollutants in rural air are extremely variable and not associated with time of day or season.

Toxic air factors and relative humidity

Druett & May (1968) suggested that rapid fluctuation of relative humidity such as occurs in open air might contribute to the adverse effect it has on microbial

survival. They failed to demonstrate any effect of artificial fluctuation in laboratory air.

During the present series of tests an instrument was used to record rapid changes in wet-bulb depression (W. C. Wright, personal communication). It was found that the rapid changes in relative humidity occurring in open air were not reflected inside the sphere (Fig. 6). Measurements made in several positions in the ventilated

Table 2. Escherichia coli *survival in ventilated aerosols and on microthread held simultaneously in open air*

Aerosol age (microthread exposure time)			
5 min		60 min	
% viability			
(*a*)	(*b*)	(*a*)	(*b*)
74	68	9	3
51	69	6	12
84	89	16	42
86	85	10	20
90	89	12	14
75	80	5	14
87	88	4	27
78	57	6	2
68	43	3	2
45	69	1	3
78	57	6	2
46	57	4	27
71	77	2	4
80	61	4	8
70	52	12	22
Average values			
72	69	7	13

(*a*) Aerosol particles collected in bottom stage of multistage sampler from ventilated aerosols in sphere.

(*b*) Particles held on microthread in open air.

sphere, including one within a few feet of the air intake, gave similar answers. Since the EC exposed on microthread survived similarly under both conditions of relative humidity, i.e. in the ventilated sphere and in open air, it was concluded that rapid changes in relative humidity had no apparent effect on the survival of EC.

Survival curves of EC in ventilated and non-ventilated aerosols were obtained at various relative humidities within the 40–95% range as indicated by conventional wet and dry bulb thermometer readings. These were compared to determine whether any correlation existed between the effect of open air factors and relative humidity. The results (Fig. 7) showed that in the absence (non-ventilated) of open air factors EC survival was not significantly affected at relative humidities of 71–95% but was adversely affected at lower relative humidities. It was found that the adverse effect of 'open air' could be large irrespective of the relative humidity.

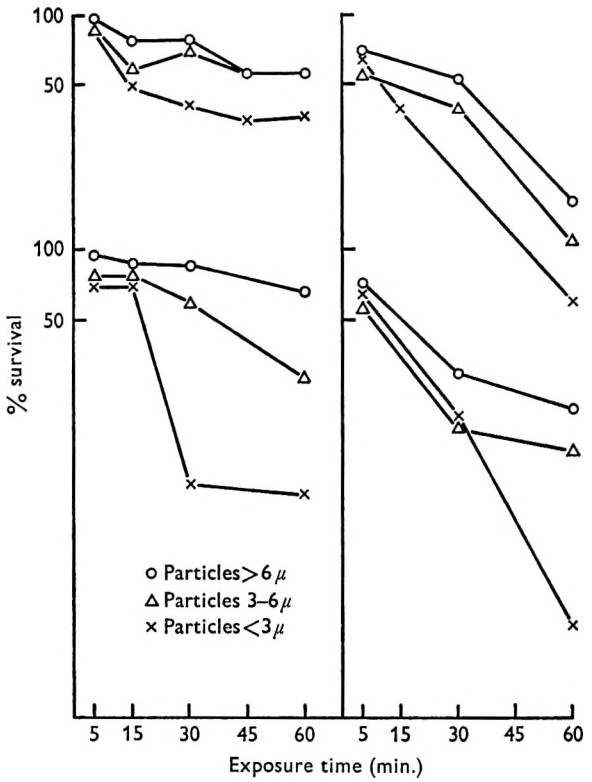

Fig. 5. *E. coli* survival in aerosols ventilated in spheres: effect of particle size.

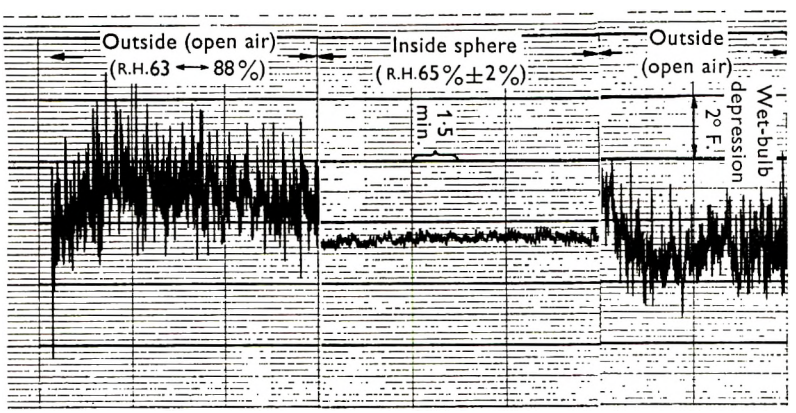

Fig. 6. R.H. fluctuation inside sphere and outside (open air).

Tests with air-intake tube

The possibility of using ducted air for ventilation of the sphere was examined because it afforded the distinct advantage of a controlled air intake pathway. The work of Druett & May (1968) indicated that some loss of toxic factors occurred when open air was passed through a 4·5 in. diameter tube. It was important therefore in a system designed to retain and measure the effect of unmodified open air to examine the effect of any duct applied to the air intake of the system.

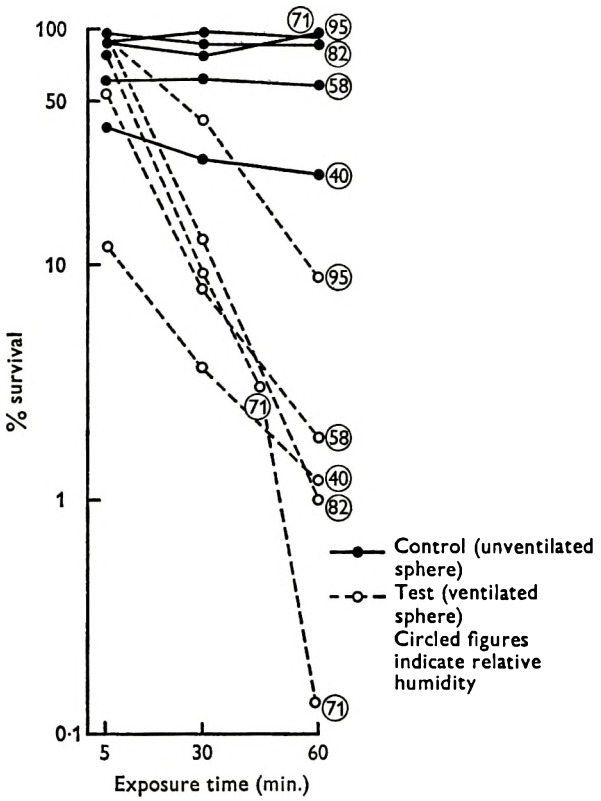

Fig. 7. *E. coli* survival in aerosols: effect of open air and relative humidity.

An air-intake tube of stainless steel 15 ft. long and 1 ft. diameter was used. This was of similar cross-section to the sphere side-arm and thus air velocity in both intake and extract situations would be similar at any given ventilation rate, thereby overcoming some of the difficulty in interpreting results associated with the adverse effect of air velocity on EC survival when held on microthread (May & Druett, 1968). The intake tube was connected from outside, through the sphere chamber wall to a stainless-steel box (*ca.* 3 × 2 × 2 ft.) which formed a connecting piece between the tube and the sphere intake orifice. The steel box was fitted with a safety-glass panel, gloves and an airlock to afford access to the inner end of the tube and to the inside of the sphere.

Viable decay curves of EC exposed on microthread were used to measure the degree of air toxicity as it passed through the system. Various rates of ventilation were used. It was found that although there was no apparent loss of toxicity in the intake tube at linear air speeds as low as 6 ft./sec. some loss occurred between this tube and the extract tube (sphere side-arm). In order to obtain air in the latter of similar toxicity to that in the intake tube an air speed in excess of 25 ft./sec. was required. This rate of ventilation corresponded to that which gave about 12 air changes/hr. in the sphere. The results indicated that the loss of toxic factors from ducted air – presumably due to wall losses – is slower in 1 ft. diameter tube than in the 22 ft. diameter sphere, and confirmed that in this system it is the residence

time of the air in the sphere that governs the minimum ventilation rate required to maintain the toxic factors in full throughout the system.

Further tests were made using the modified system in which EC survival in ventilated aerosols was compared with survival on microthread in open air. The results obtained at various rates of ventilation were so similar to those found previously without an air-intake tube that it was concluded that any effect of the intake tube could be disregarded as far as loss of toxicity was concerned.

DISCUSSION

The presence and activity of factors in open air toxic for EC has now been demonstrated in an indoor system. Loss of toxicity occurs quickly when air is contained and hence a high ventilation rate with fresh air is required to maintain its effectiveness. Since the loss is apparently due to removal by surfaces the size and geometry of the chamber influences the rate of ventilation required. The minimum rate indicated for the system examined is about 12 air changes/hr. for full preservation of open-air toxicity. The results obtained at various rates of ventilation suggest that the half-life of the toxic factors is about 3 min. in this particular system. This is of similar order to that found by Arnold (1959) for the phytotoxicant product from a 2-pentene-ozone reaction in a tube apparatus. This product has also been shown to be active against EC viability (Dark & Nash, 1970). The present results therefore support the indication that the as yet unidentified bactericidal pollutants in open air may be the reaction products of natural ozone and gaseous petroleum products from innumerable sources.

The most important outcome of this investigation is the discovery that the technique necessary to produce a 'natural' air condition in the sphere system examined enables aerosol tests to be made simply and with small amounts of material. Any large container is potentially suitable provided that the high removal rate of the airborne micro-organisms leaves sufficient material for effective assay over the operating period required. Since such an enclosed system removes any possible direct radiation effect of daylight the range of ambient conditions of temperature and humidity available for study is not restricted.

The results obtained strongly indicate that the ventilated system described provides a facility in which the effect of unidentified toxic factors in open air on microbial aerosols can be examined in detail. With proper safety precautions to protect laboratory personnel and thorough treatment of the air extracted from the system by filtration and heat-sterilization a safe system can be provided for work with pathogenic materials. It will thus be possible to determine the effect of open air on aerosols of pathogenic micro-organisms with particular reference to the relationship between viability (as determined *in vitro*) and respiratory infectivity measured by animal exposure.

I wish to thank I. H. Silver for his interest and advice in preparing this paper, Mrs H. I. Willis, C. B. Morris and J. Ching for technical assistance and I. Myles of the Chemical Defence Establishment, Porton, for facilitating the engineering improvisions required.

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Viricidal activity of open air

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SUMMARY

Airborne Semliki Forest virus and T coliphages were inactivated at a considerably enhanced rate in open air compared with enclosed air. Open air exerts its maximum sterilizing activity on viruses contained in the smallest sized particles.

INTRODUCTION

It is well known that viruses can remain infectious for long periods in airborne particles (Harper, 1961, 1963; De Jong & Winkler, 1964, 1968; Akers, Bond & Goldberg, 1966; Songer, 1967; Benbough, 1969, 1971). These reports show that the two principal factors that affect virus inactivation rates in the aerosol are the relative humidity of the air surrounding the infectious particles and the nature and concentration of solute content in the fluid from which the particles are generated. These results were obtained from experiments conducted in enclosed atmospheres and may therefore be of doubtful application in epidemiology. What is needed is a technique for estimating virus survival in ordinary outside air. This is difficult because stringent precautions against contaminating the habited environment must be maintained. The bactericidal activity of open air has been demonstrated by the microthread technique (May & Druett, 1968) and by the ventilated sphere technique (Hood, 1971). These methods have been used here to determine the viricidal activity of open air and compare it with bactericidal activity. The bactericidal activity has been attributed to forms of atmospheric pollution (Druett & May, 1968) and recently it has been postulated that the bactericidal agent in outside air is a gaseous complex formed by the combination of ozone, normally present in air, and gaseous unsaturated hydrocarbons such as those freely generated from internal combustion engines (Druett & Packman, 1968; Dark & Nash, 1970). It has been shown that the bactericidal activity of open air is rapidly lost during enclosure of the air; this is presumably due to the adsorption of the gaseous complex to the walls of the container (May & Druett, 1968).

The ventilated-sphere technique (Hood, 1971) has enabled us to determine the inactivation rates of viruses in open air as a function of the diameter of the particles in which the viruses are located. We consider this to be an important contribution since studies on the virus survival in particles of different size would be useful in assessing the susceptibility of animals to infection. Open wounds and

the upper parts of the respiratory system are expected to be more vulnerable to infection by organisms contained in large particles, whereas the pulmonary alveoli are accessible only to organisms contained in the smallest particles.

METHODS

Semliki Forest virus and coliphages T1 and T7 were grown as described before (Benbough, 1971). *Escherichia coli* MRE 162 was grown as described by Cox (1966). *Bacillus subtilis* var. *niger* spores were used as a tracer to allow for the physical decay of aerosols. The suspending fluids and the microbial assays have been described before (Cox, 1966; Benbough, 1971).

The microthread technique

The aerosols generated by a Collison spray and deposited on microthreads (May & Druett, 1968) were exposed to the open air and shaded from direct sunlight. Microthreads to which arbovirus was attached were kept near the mouth of a duct through which open air was drawn (May, Druett & Packman, 1969). This is a device used when potentially pathogenic micro-organisms were tested.

The ventilated sphere

This system has been used to determine the survival of microbes in true aerosols in open air. The sphere itself is a metal chamber of 22 ft. diameter from which aerosol is sampled by means of a tubular appendage, the whole system being usually operated in a closed state. In its ventilated role outside air is drawn through the sphere at such a rate that loss of germicidal activity due to enclosure is overcome. The minimum ventilation rate required to achieve this is 12 air changes/hr. and in practice the rate used is 14 changes/hr. The rates apply to tests of both bacteria and viruses.

A suspension containing approximately 2×10^{11} p.f.u. of the test virus, 2×10^{11} *Bacillus subtilis* spores and 2·4% (w/v) solutes in 200 ml. of fluid was completely aerosolized within 5 min. by means of a fluid atomizer (May, 1966) at the centre of the sphere. A wide range of particle sizes are produced by this spray. Aerosol samples were taken at intervals using the three-stage liquid impingers (May, 1966) which separate aerosol particles into three fractions according to size ($< 3\,\mu$m., 3–6 μm. and $> 6\,\mu$m. in diameter).

Usually the viricidal effect of open air was tested by microthreads and the sphere concurrently. For the purpose of comparison with bactericidal activity the survival of *Escherichia coli* MRE 162 on microthreads exposed to the same air was measured.

RESULTS

Microthread tests

The decay rates of *E. coli* MRE 162 held on microthreads and exposed to open air varied enormously from day to day (Fig. 1). These decay rates ranged from 0·3% min.$^{-1}$ to 5·0% min.$^{-1}$ over a 1 hr. exposure period in relative humidities

between 75% and 96% and temperatures between 2° and 12° C. The decay rate in enclosed atmospheres in these conditions never exceeded 0·2% min.$^{-1}$.

T1 and T7 coliphages on microthreads also had enhanced decay rates in open air compared to enclosed air. However, unlike those of bacteria, decay rates of the coliphages did not vary greatly from day to day. For example, when T1 coliphage was exposed on 40 different days to open air the decay rates were within the range of 1·0% min.$^{-1}$ to 1·4% min.$^{-1}$ for a 1 hr. exposure period (Fig. 2*a*). A similar type of result was found when T7 coliphage was tested (Fig. 2*b*).

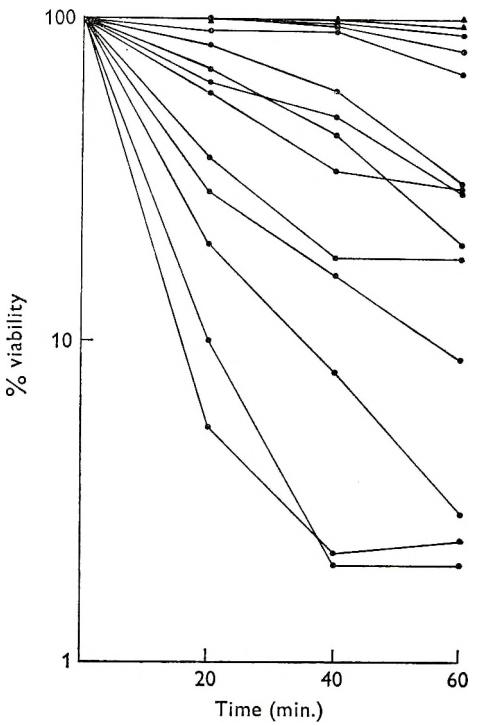

Fig. 1. The decay rates of *Escherichia coli* MRE 162 attached to microthreads and exposed to the open air on different days (all experiments were conducted at temperatures from 2° to 12° C. and relative humidity 75–96%). ▲, Microthreads in enclosed air; ●, microthreads exposed to open air.

When Semliki Forest virus was held on microthreads inactivation of the virus occurred at a rate of 2% min.$^{-1}$ even in enclosed air at relative humidities of 75% and above. This is atypical of the survival characteristics of Semliki Forest virus in true airborne particles. For example, in a rotating drum at these conditions, the rate of inactivation did not exceed 0·1% min.$^{-1}$ (Benbough, 1969, 1971, also see later for non-ventilated sphere data). Therefore, the microthread technique cannot be considered ideal for simulating Semliki Forest virus in true aerosols. Nevertheless experiments which were done using microthreads showed that Semliki Forest virus was inactivated at a much faster rate in open air (10% min.$^{-1}$) than in enclosed air (2% min.$^{-1}$) (Fig. 3). Again it was found that the day-to-day variation in the inactivation rates in open air was small.

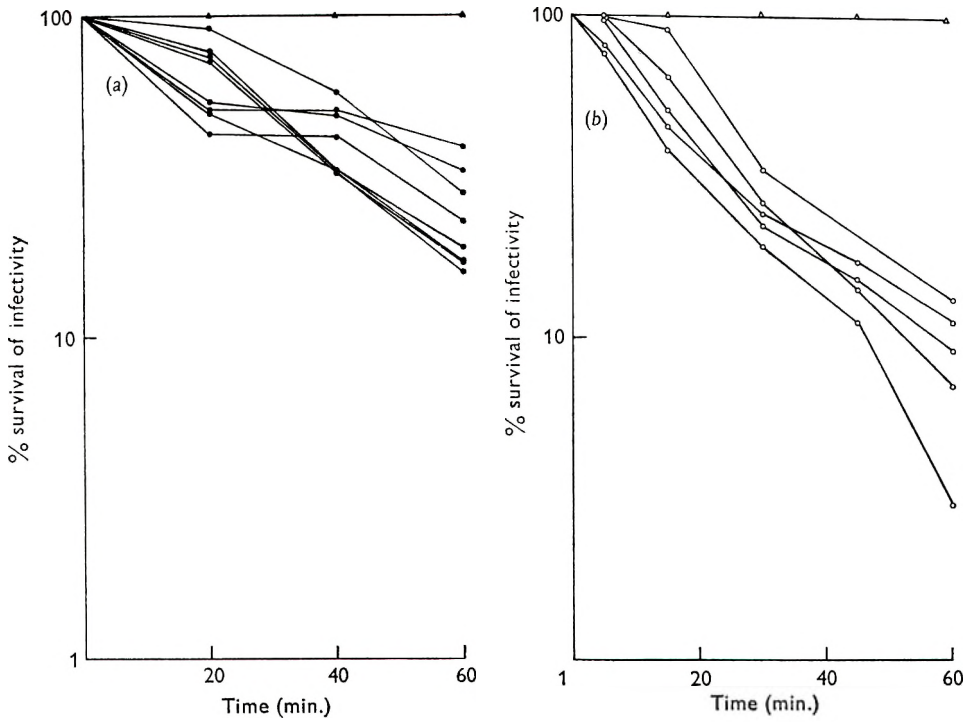

Fig. 2(*a*). The inactivation rates of T1 coliphage attached to microthreads and exposed to open air (all experiments were conducted at temperatures from 2° to 12° C. and relative humidity 75–96%). ▲, Microthreads in enclosed air; ●, microthreads exposed to open air. (*b*) The inactivation rates of T7 coliphage attached to microthreads and exposed to open air (between 2° and 12° C. and 75–96% relative humidity). △, Microthreads in enclosed air; ○, microthreads exposed to open air.

Ventilated-sphere tests

Because the microthread technique is inexplicably unsuitable for simulating aerosols containing Semliki Forest virus, the use of the sphere to measure the viricidal activity of open air assumes greater importance. Many tests (20) conducted in the ventilated sphere showed that the rate of Semliki Forest virus inactivation ranged from 1·3% min.$^{-1}$ to 1·5% min.$^{-1}$ in aerosols at relative humidities between 75% and 95% and temperatures between 2° and 12° C. Over the same storage period in the closed (non-ventilated) sphere the inactivation of Semliki Forest virus is approximately 0·1% min.$^{-1}$. Therefore the ventilated sphere experiments show a considerable viricidal activity of open air which is fairly constant from day to day (Fig. 4). The results also show that the inactivation rate of Semliki Forest virus has an inverse relationship to the diameter of the airborne particle in which the virus is contained. Fig. 5 shows that this relationship also applies to airborne T1 coliphage.

In Fig. 6 is shown the decay rates of *E. coli* MRE 162 (measured on microthreads) and of Semliki Forest virus (in the ventilated sphere) plotted against each other

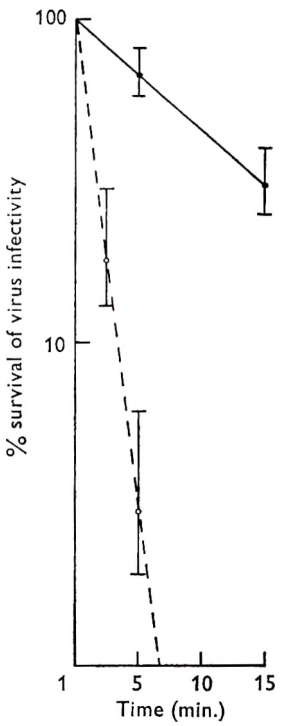

Fig. 3

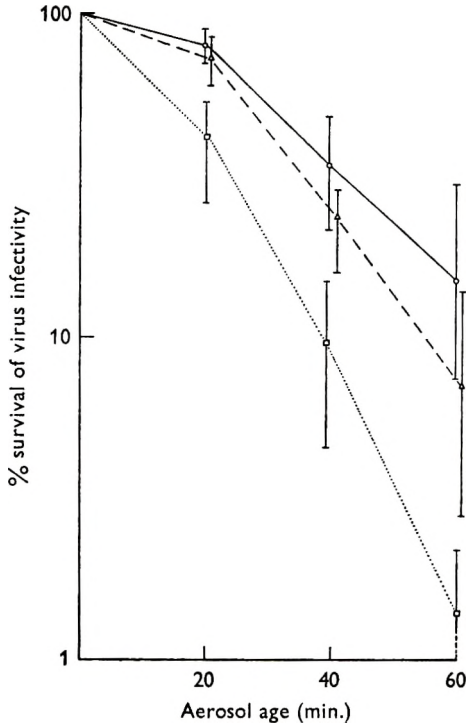

Fig. 4

Fig. 3. The inactivation of Semliki Forest virus on microthreads. ●, In enclosed air; ○, in open air.

Fig. 4. The inactivation of Semliki Forest virus in different sized particles in the ventilated sphere. ○, Virus contained in particles over 6 μ in diameter; △, particles between 6μ and 3μ in diameter; □, particles less than 3 μ in diameter. Each point is an average of 20 experiments carried out on different days at temperatures from 2° to 12° C. and relative humidities from 75 to 96%.

for each day that both were exposed. This plot shows that the viricidal activity is constant from day to day whereas the bactericidal activity varies considerably.

DISCUSSION

The large day-to-day differences in the decay rates of airborne *E. coli* MRE 162 could be accounted for by large variations in the concentration of an unknown bactericidal atmospheric pollutant (May & Druett, 1968; Dark & Nash, 1970). The concentration of pollutant surrounding the bacteria under test will depend upon the distance between the pollutant source and test areas and the direction of the wind. Also, if the pollutant is unstable the wind speed between the source and test areas would be important. The constant viricidal activity of open air suggests the possibility that this is caused by another pollutant whose concentration is constant regardless of the meteorological conditions.

Alternatively airborne viruses and bacteria may be inactivated by the same component in open air but may be susceptible to different concentration ranges.

Fig. 7 shows the kind of response to be expected if bacterial decay gradually increases with increase in concentration of airborne pollutant whereas maximum virus decay is caused by traces of the same component that are invariably present in open air.

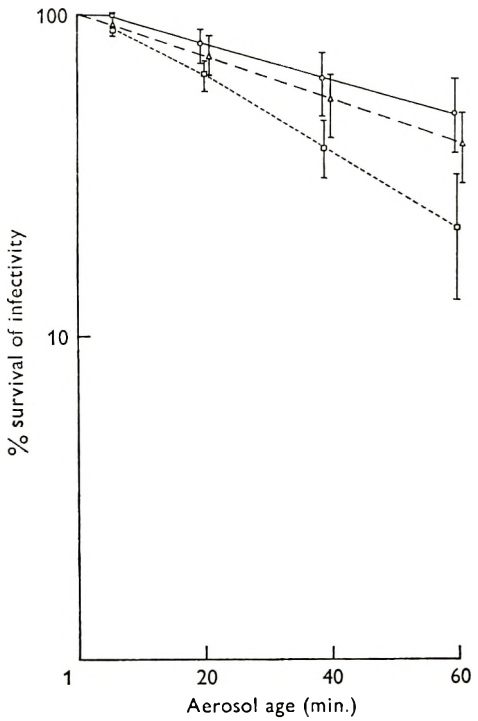

Fig. 5. The inactivation of T 1 coliphage in different-sized particles in the ventilated sphere. ○, Phage in particles over 6 μ diameter; △, in particles between 6 μ and 3 μ; □, in particles less than 3 μ. Each point represents an average of four experiments on different days at temperatures between 7° and 12° C. and relative humidities between 78 % and 92 %.

The ventilated-sphere method has yielded data on the survival of viruses in particles of different sizes that have been previously lacking in the literature. In enclosed air no differences in infectivity related to particle size can be detected. In open-air conditions a particle size effect is observed. If the airborne component attacks through the surface of particles containing virus then the simplest hypothesis is that virus inactivation rate is directly proportional to the surface to volume ratio of the particle; that is, $\pi d^2/\pi d^3/6$, which is to the inverse of the particle diameter, d.

The marked viricidal activity of open air alters predictions for survivals of normally occurring animal viruses in open air. For example, the airborne route has been strongly implicated as the cause of cross-infection of foot-and-mouth disease virus (Hyslop, 1965; Henderson, 1969; Sellers & Parker, 1969). In enclosed air this virus behaves in the aerosol like poliovirus (De Jong & Winkler, 1968), Columbia SK group virus (Akers *et al.* 1966) and T-coliphages (Benbough, 1971)

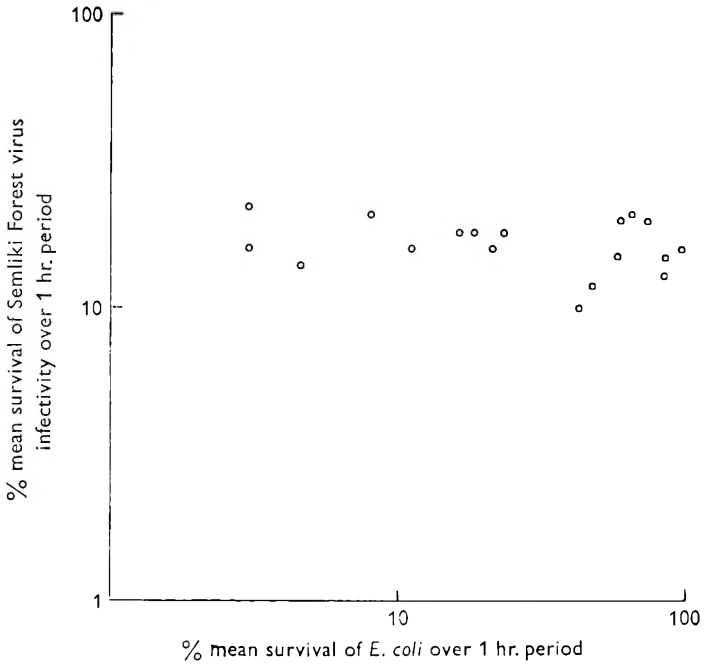

Fig. 6. The variable bactericidal effect compared to the constant viricidal effect of open air on different days. (The % mean survival over 1 hr. is defined as the mean survival of organisms at 20 min., 40 min. and 60 min.)

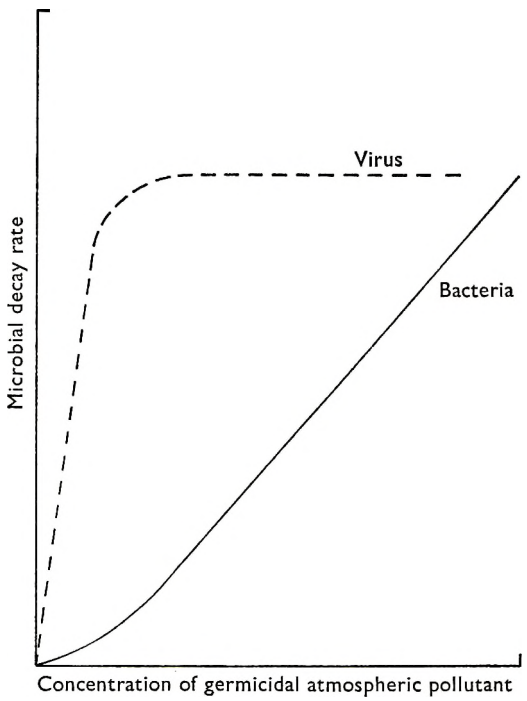

Fig. 7. The two different possible responses of the decay rates of airborne bacteria and viruses to concentration of atmospheric pollutants.

in that it survives well only at relative humidities of 70% or over (G. J. Harper, personal communication). The viricidal activity of the agent present in open air may alter Sellers & Parker's (1969) calculation on the persistence of infective foot-and-mouth virus downwind in cold humid conditions by a considerable amount. Assuming the susceptibility of this virus's infectivity to the agent to be similar to viruses tested here, then its persistence in similar conditions would be considerably lowered.

We are grateful to Dr K. P. Norris, Dr P. Hambleton and Mr G. J. Harper for helpful advice and to Mrs H. I. Willis, Mr S. Thompson and Mr J. Ching for technical assistance.

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Study of an attenuated strain of feline infectious enteritis (panleucopaenia) virus

I. Spread of vaccine virus from cats affected with feline respiratory disease

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SUMMARY

In the course of developing a living attenuated feline infectious enteritis (panleucopaenia) vaccine, it was found that respiratory disease-infected cats newly inoculated with this vaccine spread vaccine virus to respiratory disease-infected in-contact controls. These in-contact controls were able to infect other cats with which they were placed in contact so that after five natural transmissions in this way and two oral administrations and subsequent re-isolations, reversion to virulence became evident. It is clear that before general release of a new living feline infectious enteritis vaccine, there must be satisfactory evidence that concurrent infection will not affect the safety of the modified antigen.

In cats infected with feline infectious enteritis there appears to be a short period, coinciding with the onset of leucopaenia, during which they are highly infectious. It seems possible that some infected animals may become immune carriers because virus has been recovered from the small intestine of two of four cats with significant antibody titres 22–24 days after exposure to infection.

INTRODUCTION

During preliminary investigations with a living attenuated feline infectious enteritis (FIE) vaccine prepared in a feline embryonic cell line (O'Reilly & Whitaker, 1969), no spread of virus from 11 vaccinated cats to 21 in-contact controls occurred. In later work, however, evidence of contact spread was obtained at a time when a moderately severe outbreak of respiratory disease was also present. This paper reports the results of investigations into the role of feline respiratory disease in the spread of an attenuated FIE vaccine virus and the possibility of reversion to virulence by successive cat to cat passage.

MATERIALS AND METHODS

Cats

Details of the cat colonies, method of obtaining sera and measurement of neutralizing antibody have been described by O'Reilly, Paterson & Harriss (1969).

FIE vaccine

A wild strain of FIE virus, recovered from the kidneys of an affected cat, was adapted to grow in a feline embryonic cell line (O'Reilly & Whitaker, 1969) and, after serial subculturing, was shown to have lost its pathogenicity, although it was still immunogenic for cats. In Expt. 1 the vaccine consisted of undiluted virus-infected tissue culture fluid and cells. In Expt. 2, similar material was diluted with stabilizer in the proportion of 3:2 and freeze-dried.

Virus isolation

Cat tissues for examination were ground with sterile sand in a mortar and a 10% suspension prepared in sterile phosphate buffered saline containing 2000 units of penicillin and 1000 μg. of streptomycin per ml. After centrifuging at 2000 *g* for 30 min., the supernatant was collected and stored at $-20°$ C. until required. Tenfold dilutions in 0·25 ml. volumes were inoculated into each of six test tubes containing a coverslip and $2{\cdot}0 \times 10^5$ freshly versenized feline embryonic cells in 2·0 ml. of medium. The cells were incubated in the stationary position at 37° C. One coverslip per dilution was removed each day, stained and examined for evidence of FIE infection (O'Reilly & Whitaker, 1969).

Respiratory disease viruses

The feline picornavirus (K-3/C1) and the feline herpes virus (F-62/C1/1) used in these experiments had been plaque purified and were free of contaminating FIE virus. Infection of cats was done under general anaesthesia by spraying 0·5 ml. of a 1/2 dilution of virus into each nasal passage.

Experimental procedures

Experiment 1. *Contact spread.* Twenty-four kittens, aged from 8 to $10\frac{1}{2}$ weeks, from six litters bred at the Wellcome Veterinary Research Station, Frant, were randomly divided into four equal groups. Group A was infected with the feline picornavirus and group B with the feline herpes virus. These two groups were kept in separate rooms in strict isolation. The attendants wore protective rubber clothing which was washed down with 'Tego'* before entry and after leaving each of the rooms. Group A kittens were always visited before group B kittens.

Groups C and D, which were housed in another isolation building, were not infected with respiratory disease and there was no contact between personnel attending these two groups and groups A and B. Three kittens in each of groups A, B and C were inoculated subcutaneously once with attenuated FIE vaccine. The remaining kittens were unvaccinated and served as controls to the vaccine. Three days after vaccination, three group D kittens were placed in contact with group B, and 5 days after vaccination the other three group D kittens were placed in contact with group A. Rectal temperatures were recorded daily.

Experiment 2. *Reversion to virulence by cat passage.* Cats used in this experiment

* Tego MHG, Hough, Hoseason Company Ltd., Manchester.

had been bred at the Wellcome Research Laboratories and were aged 14–26 weeks. Five cats, immediately after inoculation with freeze-dried attenuated FIE vaccine, and two unvaccinated cats, were infected with the feline herpes virus and housed together in one room of an isolation building. A further two unvaccinated cats also infected with this feline herpes virus were introduced every 5th day until the 35th day of the experiment. On the 10th and 15th days two cats, unvaccinated and not infected with respiratory disease, were also added.

The vaccinated cats were killed on the 15th day and the unvaccinated cats were killed after they had been in the experiment for periods varying from 16 to 27 days. Whenever possible, serum was obtained from all cats at the time of death when bone marrow, mesenteric lymph node and portions of duodenum, ileum and spleen were also collected for recovery of virus.

RESULTS

Experiment 1. *Contact spread*

Group A. Only one of the six kittens developed a temperature exceeding 105° F. and this persisted from the 3rd to the 7th day. The three kittens from group D became pyrexic 5–8 days after being introduced. Evidence of respiratory disease was seen in three of the six group A kittens and in two of the three group D in-contact kittens. Symptoms were mild although little food was eaten by the group A kittens during the first few days of the experiment. Some of the kittens had slight inflammation of the borders of the tongue.

Group B. One kitten died within 24 hr. of infection. The remaining five developed temperatures of about 105° F. on one or more occasions between the 3rd and 9th day. Two of the group D kittens developed temperatures between the 8th and 11th day after being placed in contact.

The group B kittens showed no interest in food for the first 5 days and became severely affected with respiratory disease. One was destroyed on the 13th day because of its poor condition and two had glossitis. Symptoms in group D kittens were milder.

Clinical respiratory disease caused by the herpes virus was more severe than that produced by the picornavirus. However, all kittens infected with either of the two viruses lost weight.

Group C. These kittens remained free of respiratory disease and maintained normal temperatures.

Post-mortem findings. All the surviving kittens were bled and destroyed on day 21 of the experiment. Nothing of significance was seen in the group A kittens or in those in contact with them. In group B, foci of red hepatization were found in the cardiac lobes of the lungs of three of the four remaining kittens. Similar lesions were seen in the lungs of the in-contact group D animals. No abnormalities were seen in the group C kittens.

Antibody to FIE virus. At the start of the experiment, no kitten had demonstrable FIE antibody. When the surviving kittens were bled on the 21st day, antibody was found in five of the eight vaccinated kittens in groups A, B and C,

in four of the five animals in groups A and B which were unvaccinated but infected with respiratory disease, and in two of the three group D kittens in contact with group A. There was no rise of FIE antibody in the three unvaccinated kittens in group C which were not infected with respiratory disease nor in the three group D kittens in contact with group B (Table 1).

Table 1. *The titres of FIE antibody found in both vaccinated and in-contact unvaccinated cats infected with feline respiratory disease* (*Expt.* 1)

Group	Treatment	Antibody titres	
		Day 0	Day 21
A	Vaccine plus feline picornavirus	<8*	128
		<8	128
		<8	<8
	Feline picornavirus only	<8	512
		<8	512
		<8	128
D	In-contact with group A from the fifth day after vaccination	<8	512
		<8	128
		<8	<8
B	Vaccine plus feline herpes virus	<8	128
		<8	128
		<8	Died
	Feline herpes virus only	<8	128
		<8	<8
		<8	Died
D	In-contacts with group B from the third day after vaccination	<8	<8
		<8	<8
		<8	<8
C	Vaccine	<8	128
		<8	<8
		<8	<8
	No vaccine	<8	<8
		<8	<8
		<8	<8

* Reciprocal of serum dilution.

Experiment 2. *Reversion to virulence by cat passage*

Only mild signs of respiratory disease were observed during the first four weeks; thereafter the symptoms increased in severity.

No cat had antibody to FIE virus at the beginning of the experiment. With two exceptions, all had developed significant titres by the time they were re-examined (Table 2). The exceptions were a cat that died of a *Pasteurella* pericarditis after 13 days exposure, and another that died from pulmonary oedema after only four days. From the latter (cat 27), FIE virus was recovered from the mesenteric lymph node, spleen and bone marrow. It was also recovered from either the small intestine or mesenteric lymph node of five other unvaccinated cats fouund ead or killed 13–24 days after introduction to the experiment (Table 3).

Table 2. *The day of introduction, duration of exposure and final antibody titre of cats used in Expt. 2: reversion to virulence by cat passage*

Cats (number)	Day of introduction	Duration of exposure	Final antibody titre†
Vaccinated			
3	0	15	512‡
5	0	15	512
7	0	15	512
8	0	15	512
10	0	15	512
Unvaccinated			
1	0	20	512
6	0	20	512
11	5	20	128
12	5	13	Died
13	10	20	128
16	10	20	128
14*	10	25	128
15*	10	25	128
17	15	20	128
18	15	20	512
20*	15	27	128
23*	15	27	128
25	20	16	128
26	20	22	512
27	25	4	Died
29	25	24	32
30	30	19	128
35	30	19	512
41	35	22	512
43	35	22	512

* These cats were not deliberately infected with feline respiratory disease.
† All cats were devoid of antibody at the beginning of the experiment.
‡ Reciprocal of serum dilution.

Table 3. *Recovery of FIE virus from the tissues of vaccinated and in-contact cats and their antibody titres at the time of death (Expt. 2)*

	Days after vaccination or exposure									
Tissue	4	13	15	16	19	20	22	24	25	27
	(1)	(1)	(5)*	(1)	(2)	(7)	(3)	(1)	(2)	(2)
Duodenum	0/1†	0/1	0/5	0/1	0/2	0/7	1/3	0/1	0/2	0/2
Ileum	—	—	—	0/1	—	0/1	—	1/1	—	—
Mesenteric lymph node	1/1	1/1	—	1/1	1/2	0/2	0/3	—	0/1	—
Spleen	1/1	0/1	—	0/1	0/1	0/2	0/1	—	0/2	—
Bone marrow	1/1	0/1	0/5	0/1	0/2	0/7	0/3	0/1	0/2	0/2
Antibody titre-range	NA	NA	512‡–512	128	128–512	128–2048	512–512	32	128–128	128–128

NA = Not available.
( ) = number of cats examined.
* Vaccinated cats.
† Numerator = number of isolations of virus; denominator = number of cats providing tissue.
‡ Reciprocal of serum dilution.

One ml. of the pooled supernatants from the suspensions of spleen, bone marrow and mesenteric lymph node of cat 27 was administered orally to one of the two cats infected with the feline herpes virus and sharing the same cage. Daily leucocyte counts were done and both cats were killed on the 15th day. The orally

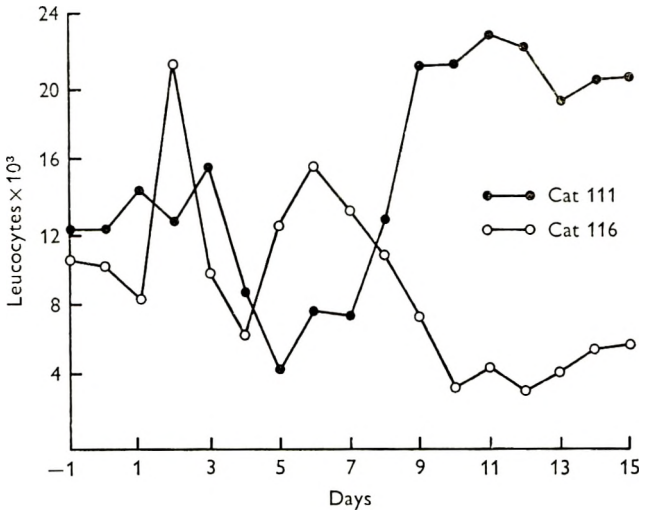

Fig. 1. Daily leucocyte counts of cat 111, dosed orally with a suspension of virus-infected tissues from cat 27, and of cat 116, the in-contact control (Expt. 2).

Table 4. *Isolations of virus from, and antibody titres of, cats* 111 *and* 116 (*Expt.* 2)

	Cats	
Tissues	111	116
Ileum	0	+
Faeces	0	+
Mesenteric lymph node	0	+
Spleen	NT	+
Bone marrow	NT	0
Antibody titres		
Day 0	<8*	<8
15	512	32

+ = virus isolated.
0 = no virus isolated.
NT = Not tested.
* Reciprocal of serum dilution.

dosed cat (111) was leucopaenic on the 5th day and its in-contact cage-mate (116) was similarly showing leucopaenia on the 10th to 13th days (Fig. 1). While no virus was recovered from tissues taken after death from cat 111, virus was isolated from the mesenteric lymph node, ileum, faeces and spleen of cat 116. Both cats developed antibody (Table 4).

Two cats (70 and 73) infected with the herpes virus were each infected orally with 1 ml. of a suspension prepared from the tissues of cat 116. On each of days

5 and 11, two cats, not infected with respiratory disease, were introduced. Cats 70 and 73 were killed on the 10th day and the in-contact animals between the 16th and 28th days. Daily leucocyte counts showed that all the cats developed leucopaenia (Fig. 2) and they lost both appetite and weight, shortly after entry

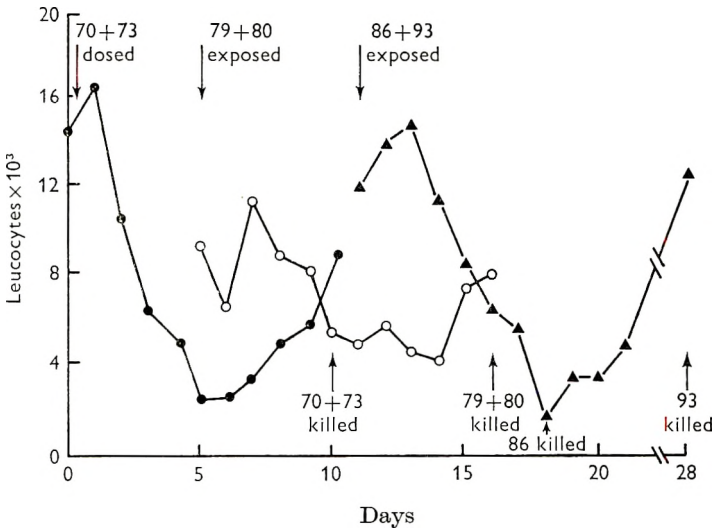

Fig. 2. Geometric mean daily leucocyte counts of two cats dosed orally with a suspension of virus-infected tissues from cat 116, and of four cats in which the virus was passaged naturally (Expt. 2).

Table 5. *Antibody titres of two cats after oral infection with a suspension of tissues from cat* 116 *and of the four cats in which the virus was subsequently passaged naturally* (*Expt.* 2)

	Days after dosing or exposure				
	0	7	11	12	17
Dosed cats					
70	< 8*	—	128	—	—
73	< 8	—	8	—	—
Exposed cats					
79	< 8	—	—	32	—
80	< 8	—	—	32	—
86	< 8	< 8	—	—	—
93	< 8	—	—	—	32

* Reciprocal of serum dilution.

into the experiment. The weight loss, although appreciable in cats 70 and 73, became more noticeable in each new entry even though none of the cats were clinically affected with respiratory disease. The last pair of cats added were vomiting on the 5th day of exposure and one (cat 86) was killed on the 7th day to provide tissues for recovery of virus. FIE virus was isolated from its mesenteric lymph node, jejunum, spleen and urine. No attempts were made to recover virus from the tissues of the other cats. With the exception of cat 86, all developed neutralizing antibody (Table 5).

DISCUSSION

The findings reported in this paper add a further requirement to the tests used in the development of a living FIE vaccine. Before general release, there must be satisfactory evidence that concurrent infections will not affect the safety of the modified antigen.

Expt. 1 showed quite clearly that the FIE attenuated vaccine virus used in these studies spread to in-contact control kittens in the presence of concurrent respiratory disease, despite the fact that some animals showed no evidence of clinical respiratory symptoms. In the absence of deliberate concurrent infection with one or other of the respiratory viruses, this did not occur (group C).

Table 6. *Proportion of* 8- *to* $10\frac{1}{2}$*-week-old kittens developing FIE antibody* 3 *weeks after either one dose of living attenuated vaccine or being in contact with vaccinated kittens* (*Expt.* 1)

Age in weeks at time of vaccination	Proportions developing antibody	
	Vaccinated	Unvaccinated
$10\frac{1}{2}$	1/2	2/6
10	1/3	2/5
$9\frac{1}{2}$	2/2	2/3
8	1/1	—

Only 11 of the 22 kittens developed antibody. This is not surprising since none were older than $10\frac{1}{2}$ weeks when vaccinated (Table 6). Lack of antibody conversion in a proportion of kittens given one dose of vaccine at this age or younger has been demonstrated by O'Reilly *et al.* (1969) who were able to obtain a 100% response only in animals vaccinated at 12 weeks or older.

The results of Expt. 2 confirmed the spread of the attenuated virus from vaccinated cats to unvaccinated in-contact cats experimentally infected with respiratory virus, whether or not clinical symptoms of respiratory disease developed. Natural transmission continued over the seven contact passages carried out. When virus isolated from the fifth contact passage (cat 27) was passed twice more by oral administration and re-isolation from the recipients, clear evidence of reversion to virulence was seen in cats not infected with respiratory disease and placed in contact (cats 86 and 93; Fig. 2).

These experiments suggest that cats are highly infectious within a few days of infection and that some animals may possibly develop into immune carriers. In cats infected with FIE virus, the first indications of leucopaenia usually occur between the 4th and 6th days (Lawrence & Syverton, 1938; Hammon & Enders, 1939; O'Reilly, 1970). Presumably viraemia has preceded this event (Lawrence & Syverton, 1938) and, from Figs. 1 and 2 and O'Reilly (1970), it would appear that cats are most infectious at the onset of leucopaenia. Because antibody has been found in cats as early as 7 days after inoculation with living attenuated vaccine (O'Reilly, unpublished), it seems likely that the highly infectious period of the disease is short-lived. On the other hand, virus was recovered from the small

intestine of cats on the 22nd and 24th days after exposure (Table 3). One of these cats had an antibody titre of 32 and the other 512. However, it still remains to be confirmed that there is a 'carrier state' similar to that reported in mink infected with the related mink enteritis virus (Bouillant & Hanson, 1965).

The attenuated vaccine used in these experiments has been extensively tested in breeding catteries known to be infected with FIE where all the young kittens are vaccinated after weaning and the queens are boosted annually. No problems related either to the vaccine or to subsequent vaccination failures have been observed. However, it might not be suitable for use in boarding catteries where there is a continually changing cat population and high risk of respiratory disease, and work continues in an effort to achieve further modification and remove the propensity to spread in the presence of intercurrent infection.

I wish to thank Mr J. Prydie, M.R.C.V.S., for supplying the feline respiratory disease viruses and Mr W. F. Matchett, A.I.M.L.T., A.I.S.T., and Mrs L. M. Hitchcock for their valued technical assistance.

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Study of an attenuated strain of feline infectious enteritis (panleucopaenia) virus

II. Removal of the spread factor by further passaging in tissue culture

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(*Received* 7 *June* 1971)

SUMMARY

The propensity of an attenuated strain of feline infectious enteritis (panleucopaenia) virus to spread from vaccinated cats affected with intercurrent feline respiratory disease to unvaccinated in-contact cats was eradicated by further passaging of the vaccine virus in tissue culture. No virus was recovered from, and no antibody was found in the sentinel cats in contact with seven vaccinated animals. Thus, a further 27 passages of the vaccine virus in tissue culture has eliminated the spread factor.

INTRODUCTION

In the previous paper (O'Reilly, 1971) it was reported that an attenuated strain of feline infectious enteritis (FIE) virus, believed to be safe for use as a vaccine, spread from vaccinated cats to in-contact unvaccinated cats during an outbreak of respiratory disease. Later, it was established that the virus which was shed reverted to virulence after serial cat-to-cat passage.

This paper records the eradication of the spread factor by further modification of vaccine virus.

MATERIALS AND METHODS

Serology

Collection of blood in Expt. 1 was by heart puncture (O'Reilly, Paterson & Harriss, 1969) and in Expt. 2 from the jugular vein (Hovell, O'Reilly, Calder & Povey, 1970): neutralizing antibody was measured by the technique of O'Reilly *et al.* (1969).

FIE vaccine

The tissue culture adapted strain of virus used in the previous experiments (O'Reilly, 1971) was serially subcultured a further 27 times in a feline embryonic cell line (O'Reilly & Whitaker, 1969). In Expt. 1, vaccine consisted of undiluted virus-infected tissue culture fluid and cells, the dose being 1·0 ml. In Expt. 2, similar material was diluted with stabilizer in the proportion of 3:2 and given as a 2·0 ml. dose.

Respiratory disease virus

The feline herpes virus used (F-62/Cl/1) was plaque purified and free of contaminating FIE virus.

Virus isolations

The method used for attempted recovery of virus from feline tissues has been described (O'Reilly, 1971).

Challenge virus

Cats were challenged with a 10^{-2} dilution of virulent virus given orally; the characteristics of this virus pool have been described (O'Reilly, 1970).

Experimental procedures

Experiment 1. The cats, bred at the Wellcome Veterinary Research Station, Frant, were aged 17–18 weeks. Three cats were placed in contact with a donor group showing clinical evidence of respiratory disease. One week later the three experimental animals, now showing signs of respiratory disease, were moved to another isolation room where they were bled and inoculated subcutaneously with 1·0 ml. of FIE vaccine. Three healthy cats were bled, and then added to the group of vaccinated cats. Eight days later, another three healthy cats were placed in contact with the respiratory disease-infected donor group and, after 2 days, were bled and added to the experimental animals.

The animals were challenged with virulent virus on the 21st day after vaccination. White blood cell counts were done and temperatures recorded daily throughout the experiment.

Experiment 2. The cats used in this experiment were bred either at the Wellcome Veterinary Research Station (W.V.R.S.) or at these laboratories (W.R.L.). Four W.V.R.S. cats and four W.R.L. cats were placed in an isolation room; daily leucocyte counts were started 2 days later, and on the following day all eight animals were bled and infected intranasally with a feline herpes virus (O'Reilly, 1971). Two cats from each of W.V.R.S. and W.R.L. were inoculated subcutaneously with 2·0 ml. of vaccine. On each of the 5th, 10th, 15th, and 20th days after the start of the experiment, four more cats – two from W.V.R.S. and two from W.R.L. – were introduced into the environment. Only those animals added on day 5 were experimentally infected with feline herpes virus.

On the 25th day one cat introduced on the 15th day and the four introduced on the 20th day were killed and their tissues taken for recovery of virus.

On the 29th day of the experiment, all the surviving cats were bled and challenged with virulent virus.

RESULTS

Experiment 1

Clinical

Throughout the duration of this experiment most cats had slightly elevated temperatures which seldom exceeded 104° F. Most cats became severely affected with respiratory disease during the first week of exposure, but thereafter the symptoms subsided and the condition became chronic with periodic recurrences of ocular and nasal involvement.

No evidence of leucopaenia or depression of the white blood cell counts was

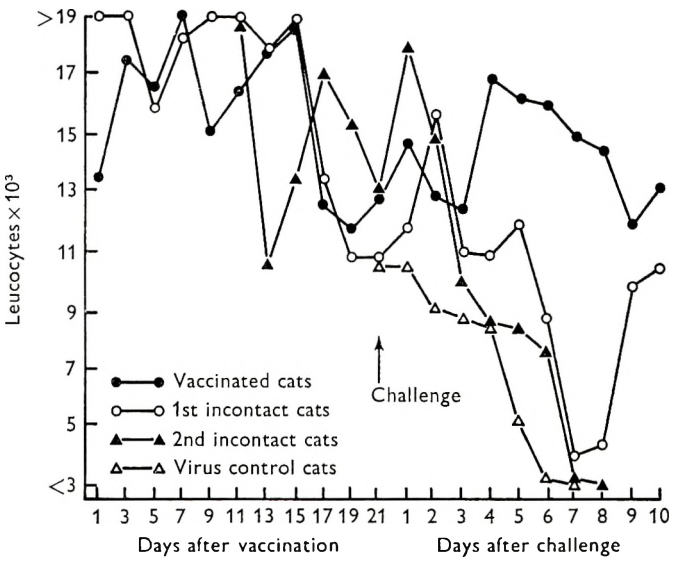

Fig. 1. The geometric means of the leucocyte counts of vaccinated, in-contact unvaccinated and virus control cats. Expt. 1.

Table 1. *SN antibody titres* (*Expt.* 1)

Treatment	Cat number	After vaccination (days) −14	0	3	7	10	16	21	14th day after challenge 14
Vaccinated	152	—	<8*	NB	32	NB	128	512	128
	160	—	<8	<8	8	—	—	—	—
	162	—	<8	<8	8	NB	128	128	32
In-contact controls									
1st group	153	<8	<8	NB	<8	NB	<8	<8	—
	158	<8	<8	NB	<8	NB	<8	<8	—
	159	<8	<8	NB	<8	NB	<8	<8	128
2nd group	155	—	—	—	—	<8	<8	<8	—
	157	—	—	—	—	<8	<8	<8	—
	161	—	—	—	—	<8	<8	<8	—
Virus control	154	—	—	—	—	—	—	<8	—
	168	—	—	—	—	—	—	<8	—

* Reciprocal of serum dilution.
NB = not bled.

observed between the beginning of the experiment and challenge (Fig. 1). After challenge, frank leucopaenia was seen by the 5th day in the virus control cats; both died, one on the 7th day and the other one the next day. The first group of in-contact cats did not show a drop in the leucocyte counts until the 7th day – any tendency towards an impending leucopaenia was masked by cat 153 having cell counts in excess of 19,000 cells per mm^3 during the first 5 days. Nevertheless, two of the cats in this group died on the 8th day and the 3rd cat survived. The white blood counts of the second group of in-contact controls dropped steeply from the 2nd to the 7th day and all three cats were dead by the 8th day. Neither of the two vaccinated cats showed any evidence of leucopaenia.

Antibody to FIE virus

Antibody was detected in the vaccinated cats by the 7th day and reached its peak titre by the 16th day (Table 1). Vaccinated cat 160 failed to recover from the anaesthetic after the 7th day bleeding.

Only the vaccinated cats had antibodies at the time of challenge; 14 days later, the sole surviving control cat (159) also had antibody.

Experiment 2

Clinical

Although the respiratory disease was clinically more severe than in Expt. 1 only cats bred at W.V.R.S. were affected. Two cats were killed because of the severity of the disease (cat 256 on day 13 and cat 257 on day 19), and cat 264 died of pneumonia on day 26. Vaccinated cat 254 was destroyed because it developed a physical disability suggestive of iliac thrombosis.

Between vaccination and challenge (day 29) there were no depressions of the

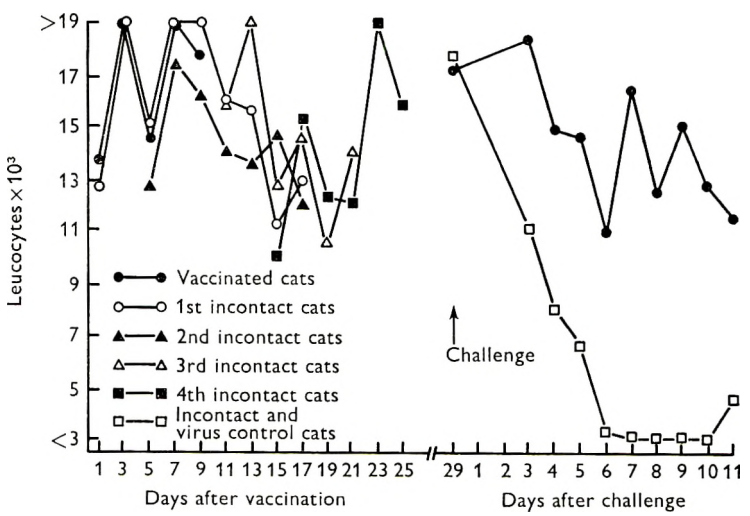

Fig. 2. The geometric means of the lencocyte counts of vaccinated, in-contact unvaccinated and virus control cats. Expt. 2.

Table 2. *SN antibody titres* (*Expt.* 2)

Treatment	Cat number	After vaccination (days) 0	5	10	11	15	20	25	29	13th day after challenge
Vaccinated	54	< 8*	NB	NB	128	128	NB	NB	128	128
	69	< 8	NB	NB	128	512	NB	NB	512	128
	253	< 8	NB	NB	128	128	NB	NB	128	128
	254	< 8	NB	NB	32	—	—	—	—	—
In-contact controls										
1st group	56	< 8	NB	NB	< 8	< 8	< 8	< 8	< 8	512
	57	< 8	NB	NB	< 8	< 8	< 8	< 8	< 8	512
	256	< 8	NB	NB	< 8	—	—	—	—	—
	257	< 8	NB	NB	< 8	< 8	—	—	—	—
2nd group	62	—	< 8	NB	< 8	< 8	< 8	< 8	< 8	512
	66	—	< 8	NB	< 8	< 8	< 8	< 8	< 8	512
	258	—	< 8	NB	< 8	< 8	< 8	< 8	< 8	—
	259	—	< 8	NB	< 8	< 8	< 8	< 8	< 8	—
3rd group	64	—	—	< 8	NB	NB	< 8	< 8	< 8	—
	66	—	—	< 8	NB	NB	< 8	< 8	< 8	512
	260	—	—	< 8	NB	NB	< 8	< 8	< 8	—
	261	—	—	< 8	NB	NB	< 8	< 8	< 8	128
4th group	80	—	—	—	—	< 8	NB	< 8	< 8	—
	81	—	—	—	—	< 8	NB	< 8	—	—
	263	—	—	—	—	< 8	NB	< 8	< 8	—
	264	—	—	—	—	< 8	NB	< 8	—	—
5th group	65	—	—	—	—	—	< 8	< 8	—	—
	82	—	—	—	—	—	< 8	< 8	—	—
	262	—	—	—	—	—	< 8	< 8	—	—
	265	—	—	—	—	—	< 8	< 8	—	—
Virus controls	271	—	—	—	—	—	—	—	< 8	512
	272	—	—	—	—	—	—	—	< 8	512
	273	—	—	—	—	—	—	—	< 8	512
	274	—	—	—	—	—	—	—	< 8	—

* Reciprocal of serum dilution.
NB = not bled.

Table 3. *Attempts at recovery of FIE virus from the tissues of cats in-contact with vaccinated cats* (*Expt.* 2)

Treatment	Cat number	Exposure in days	Tissue: Mesenteric lymph node	Bone marrow	Duodenum	Spleen	Ileum	Reason for cat death
1st group	256	13	—	—	—	—	NT	Respiratory disease
	257	19	NT	—	—	—	—	
4th group	81	10	—	NT	—	—	NT	Recovery of virus
	264	11	—	NT	—	—	NT	Pneumonia
5th group	65	5	NT	NT	—	—	NT	Recovery of virus
	82	5	—	—	—	NT	NT	
	262	5	—	NT	—	—	NT	
	265	5	—	NT	—	—	NT	

NT = not tested.
— = no virus recovered.

leucocyte counts in either the vaccinated or in-contact cats (Fig. 2). However, during the 11 days on which white cell counts were done after challenge, leucopaenia was seen in all the in-contact and virus control cats but not in the vaccinated cats.

Two specific FIE deaths occurred in each of the 2nd and 3rd in-contact control groups and one death in each of the 4th in-contact control and virus control groups; no obvious reason was found for the death of cat 263 on the 14th day after challenge.

Antibody to FIE

Antibody was found in the vaccinated cats on day 11 (first bleeding after vaccination) but none was detected in any of the other cats at challenge (Table 2). Those animals in the control groups surviving challenge all developed antibody.

Recovery of virus

Attempts to recover virus from the tissues of eight in-contact cats were unsuccessful. Four of these cats had been exposed to the experimental environment for 5 days, and the other four from 10 to 19 days. Two of the cats, destroyed because of respiratory disease, were from the first group of in-contact animals (Table 3).

DISCUSSION

The previous paper (O'Reilly, 1971) stated that FIE virus passaged in tissue culture might appear to be fully attenuated because there was no spread of vaccine virus from healthy vaccinated cats to healthy in-contact cats. However, cats suffering from intercurrent feline respiratory disease were capable of shedding vaccine virus which reverted to virulence after several cat-to-cat passages.

This paper reports that the vaccine strain of virus referred to above has, after further passaging in tissue culture, become fully attenuated without loss of antigenicity. Intercurrent infection of cats with respiratory disease did not provoke spread of vaccine virus since there was no development of antibody in in-contact cats, no isolation of virus from cats either severely affected with respiratory disease or killed shortly after potential exposure to virus and no depression of the leucocyte counts.

After challenge, none of the five vaccinated cats showed any signs of ill health whereas 58% (14/24) of the in-contacts and virus control cats died of the disease; the surviving 42% of these cats all had antibody conversions. It has been demonstrated by O'Reilly (1970) that cats with detectable antibody do not show leucopaenia or depression of leucocytes after challenge with virulent virus. Conversely, he showed that cats without antibody at the time of challenge become leucopaenic or exhibit a depression in the number of circulating white blood cells and the surviving animals develop antibody.

This paper confirms the necessity for testing new living vaccines for contact spread of vaccine virus to animals afflicted with some intercurrent infectious disease. In such conditions, the test animals are likely to be under greater strain

than healthy laboratory animals, a situation more closely resembling the natural environment.

I am indebted to Mr J. Prydie, M.R.C.V.S., for the feline herpes virus and to Mr W. F. Matchett, A.I.M.L.T., A.I.S.T., and Mrs L. M. Hitchcock for their invaluable technical assistance.

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Cytomegalovirus antibody production in renal transplant patients

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SUMMARY

Sera were examined from 50 patients on the renal transplant unit, Cambridge, for antibody against cytomegalovirus by complement fixation and by immunofluorescence for IgG and IgM antibodies.

The incidence of antibody on admission was 84% with a possible further 8% so that nearly all had been infected at some time by CMV.

43 (86%) patients showed evidence of active infection after admission, 39 by serology and four only from the examination of post-mortem material.

Twenty-one patients produced IgM antibody and production was prolonged for years in patients that survived. Antibody production was related both to transplantation and admission to hospital.

The evidence indicated that primary CMV infections were rare, that IgM antibody production was the result of active infection and that this could be attributed to reactivation without the need to invoke re-infection as the source although this type of patient is both susceptible and exposed to re-infection.

INTRODUCTION

The introduction of immunosuppression by the combination of corticosteroids and azathioprine, an analogue of 6-mercaptopurine, to reduce the rate of rejection of organ transplants was followed by an increase in the incidence of cytomegalovirus (CMV) infections in the recipients (Hedley-Whyte & Craighead, 1965).

It was soon found that other herpesviruses caused severe infections in renal transplant patients, e.g. varicella-zoster (Rifkind, 1966) and herpes simplex virus (Montgomerie *et al.* 1969) and as herpesviruses are known to produce latent infection the possibility of reactivation arose (Kanich & Craighead, 1966). However, immunosuppression also leads to increased susceptibility to infections of many kinds, including primary CMV infections (Bodey, Wertlake, Douglas & Levin, 1965) so that the problem arose of the part played by the two sources of virus, reactivated endogenous virus (Kanich & Craighead, 1966) compared with fresh infection (Craighead, Hanshaw & Carpenter, 1967). The demonstration that the post-perfusion syndrome (Kääriäinen, Klemola & Paloheimo, 1966) in open-heart surgery patients, not on immunosuppression, was due to CMV increased the likelihood that such CMV infections were exogenous, but even though infective

virus in donor blood was demonstrated by Kääriäinen *et al.* (1966) and others the relative parts played by reactivation and infection or re-infection continue to be the subject of discussion both in immunosuppressed (Andersen & Spencer, 1969; Armstrong *et al.* 1971) and other patients (Henle *et al.* 1970; Caul *et al.* 1971; Purcell *et al.* 1971).

As the work on renal transplant patients referred to above was based mainly on American studies it seemed of interest to compare a similar group in this country. The renal transplant unit in Cambridge has provided the opportunity and the work reported comprises a survey of the CMV antibody performance of 50 transplant recipients.

MATERIALS AND METHODS

The work described was based on the first 50 renal transplant patients from whom serum was obtained on admission, before transplantation and subsequently at intervals of about a month for an adequate period. This meant exclusion of patients that died in the early post-transplantation period before serological changes were expected to occur, so that apart from one who died after 5 weeks, but with significant serological change (M.McW.) the rest have been observed from 8 weeks to over 3 years. For simplification only the first transplant is considered, subsequent operations were not found to contribute greatly to the results obtained.

Complement fixation (CF) was performed in Perspex WHO trays by a micromethod based on Bradstreet & Taylor (1962). The antigen was the Rawles strain of CMV (Stern, Lambert & Shakespeare, 1963) grown in human embryo fibroblasts and supplied by Dr C. M. P. Bradstreet.

Fluorescent antibody (FA) staining was based on the sandwich technique used by Hanshaw, Steinfeld & White (1968), except that the Rawles strain was used instead of their A.D. 169 (Rowe *et al.* 1956) and grown in early passages of human embryo lung, the infected cells were stripped by 'Versene' 1/5000 for 5 min. followed by 0·025% trypsin for 2–3 min. then washed, transferred to slides and fixed. Twelve areas of cells per microscope slide were used so that titrations could be performed in parallel on the same slides. Sheep anti-human IgG and IgM from Wellcome Reagents Ltd. were used without absorption. Control positive and negative sera and uninfected cells were included in all titrations.

Rubella haemagglutination-inhibition (HI) titrations were done by the standard technique of Stewart *et al.* (1967) with removal of non-specific inhibitors by manganous chloride (Plotkin, Bechtel & Sedwick, 1968).

Post-mortem specimens and nephrectomy material were supplied by Dr P. D. Millard to whom I am also indebted for the histological data.

Virus isolation was attempted only from tissues, no attempt was made to examine other sources as these have been well documented by others (e.g. Craighead, 1969).

Isolation was attempted in early passage human embryo lung fibroblasts and WI-38 cells, supplemented occasionally by other fibroblasts. Sera from patients were collected by Dr D. B. Evans.

RESULTS

To establish that the anti-IgM conjugate was free of anti-IgG and vice versa high titre sera were compared and the conjugates were found to be pure.

Comparison of the mean CF titres of undiluted initial sera which produced the same degree of fluorescence of infected cells with anti-IgG, with the individual titres of the same sera showed a good correlation (Fig. 1). In other words the degree of fluorescence was proportional to the CF titre, which confirms Hanshaw *et al.* (1968) and Lang & Hanshaw (1969) who considered the CF titre of CMV antisera to be due to IgG.

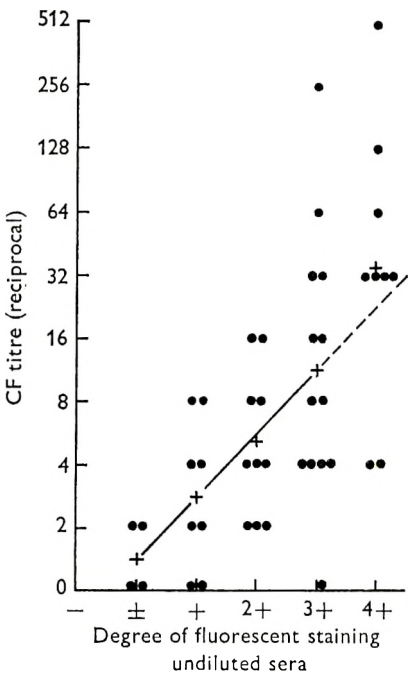

Fig. 1. Complement-fixation titre and fluorescent antibody titration of transplant patients' first sera. ●, Anti-IgG; +, mean CF titre of sera with same fluorescence.

The FA method for detection of IgG was, in general, slightly more sensitive than the CF test (Fig. 1) so that it was considered reasonable to regard a CF titre as low as 1/2 significant, provided that it was supported by specific IgG fluorescence (reading + or more on the Fig. 1 scale) and there was no anti-complementary activity. The use of CF titres of 1/2 is not usual because they can be unreliable, but because the detection of the lowest amount of antibody was essential for this study, to establish that previous infection had occurred, such titres were used with these safeguards. Earlier work in which titres of 1/2 were used include that of Craighead *et al.* (1967), Lang & Noren (1968), Andersen (1969) and Andersen & Spencer (1969).

Fluorescent antibody titres alone were considered significant provided control uninfected cells showed no fluorescence or, if they did, the titre with them was considerably less (i.e. at least eightfold) than with infected cells.

Incidence of antibody in patients

The number of patients with CMV antibody on admission was at least 42/50 (84%) and in all instances this was IgG. One patient only had a trace of IgM also. Four others had doubtful titres (Table 1).

This percentage is higher than Stern & Elek (1965) found by CF in the Greater London area in normal individuals (54%). It appears higher than in the Boston transplant patients examined by Craighead (1969) who found 41/63 (65%) with CF titres of 1/4 or more before transplantation. However if only CF titres of 1/4 or more are counted in the Cambridge patients this gives 33/50 (66%) positive which is exactly comparable, although CMV strain AD 169 was used for the American antigen. In the Boston patients the 30 to 39-year-old group was 86% positive and in our patients 84% (Table 1). Thus both series are practically identical, the main difference being a higher proportion in their under-20 years old group – 15 compared with our four patients.

Antibody titre changes

IgG antibody production. Because immunosuppression is essential after transplantation, azathioprine and prednisone were given to patients at a high dosage in the immediate post-operative period and adjusted later. This made it necessary to know if a change in titre was significant i.e. specific for CMV, or part of a general change with alteration of the immunosuppressive regime.

As a guide to general IgG production rubella HI antibody was selected because most of the patients were likely to possess it, production does not depend on the continued presence of virus, reactivation does not occur and titre changes due to transfusions were not likely to be significant.

Of the patients 47/50 (94%) had anti-rubella HI titres of 1/8 or more in their initial serum. This is similar to the incidence found in women of childbearing age in this area and as the ages of both groups are roughly the same it was felt that comparison of HI titres might show if the patients' titres were normal or not.

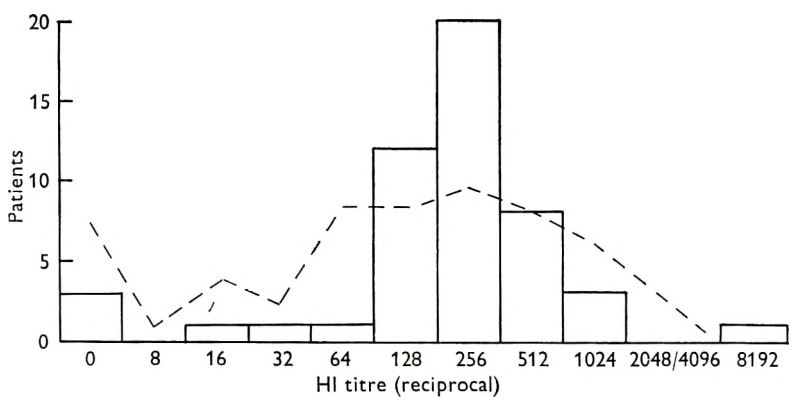

Fig. 2. Histogram of rubella HI titres of the transplant patients' initial sera. – – –, Profile of normal sera.

Table 1. *Age distribution of antibody in initial sera*

Ages	Positive: CF and FA	Positive: FA only	Negative	Total	Positive (%)
10–19	2 (1)	[1]	0	4	50
20–29	11	1	2	14	84
30–39	15 (1)	2 [1]	2	21	84
40–49	5*	1	0	6	100
50–	5	0	0	5	100
	38 (2)	4 [2]	4	50	84

( ) = two patients with CF titre 1/2, FA negative.
[ ] = two patients with FA ± reading only with undiluted serum and CF < 1/2.
These four patients are not included in the 84 % total.
* = patient with trace of IgM.

Table 2. *Range of CF titres in the 42 positive patients and IgG FA*

CF titre (reciprocal)	Patients: FA positive	Patients: FA negative	Total
0	4	6	10
2	5	2	7
4	11	0	11
8	6	0	6
16	5	0	5
32	6	0	6
64	2	0	2
128	1	0	1
256	1	0	1
512	1	0	0
Totals	42	8	50

Table 3 (*A*). *Comparison of CMV CF and Rubella HI titre changes after transplantation in all patients*

		CMV–CF: Permanent	CMV–CF: Transient	Rubella–HI: Permanent	Rubella–HI: Transient	Rubella–HI: Terminal only
Change	Rise	32	6	5	4	1
	Fall	2	0	1	9	2
No change		10	.	28	.	.

Transient change = duration less than 3 months.
Terminal change = in sera during month patient died.

Table 3 (*B*). *Patients with synchronous titre changes*

(*a*) Steady increase in CMV antibody with transient initial HI rise	3 patients (K.B., B.B., T.H.)
(*b*) Steady increase in CMV antibody with permanent fourfold HI rise	1 patient (L.D.)
(*c*) Permanent parallel small increase in both CMV (0–4) and HI (256–1024)	1 patient (R.S.)
(*d*) Transient small rise (fourfold) in both	1 patient (A.L.)

From Fig. 2 it can be seen that the initial HI titres of the patients do appear reasonably similar to those of the normal individuals.

The changes in CMV CF and rubella HI titres in the patients are shown in Table 3. Twenty-two patients had fourfold or greater HI changes but only five increases and one decrease lasted for more than 3 months. There were three patients with no detectable HI initially and two subsequently developed titres to a maximum of 1/64, the remaining patient had no detectable HI during 4 years observation. There were only six instances of parallel change in CF and HI titres (Table 3, *B*) and in five the CMV titre change was clearly significant and of a different kind to the HI. In only one patient (Table 3, *B*(*d*)) were the changes minor and equal so these were excluded from the data on titre changes.

From these results it was evident that the immunosuppressive treatment only caused minor variations in IgG production and they could readily be assessed from serial sera.

Cytomegalovirus antibody production

(*a*) *Extent of changes.* The CF changes in all 50 patients are summarized in Table 4 according to their initial IgG antibody levels. It will be seen that temporary production of a small amount of antibody was a feature of the group with little or no detectable antibody in their initial sera.

The patients that produced a considerable rise in titre did so to a ceiling of

Table 4. *CF titre changes in all* 50 *patients*

Maximum subsequent CF titre (reciprocal)	Initial CF titre (reciprocal)											
	FA−	FA+	−*	+*	4	8	16	32	64	128	256	512
1024	1				2	1	1	2				
512	1			1	1	2				1		1
256					1			1	2		1	
128		1	2	1	2		1	1				
64					1 (1)	1	2					
32	1							1				
16												
8	(1)					2		1				
4	1	(3)			3							
2				3								
0	1						1					

( ) = transient.

1/128–1/1024 which explains observations that patients with high initial titres tend to produce smaller increases (Henle *et al.* 1970; Purcell *et al.* 1971; Caul *et al.* 1971).

(*b*) *Time of change.* To try to see which events might be associated with CMV antibody production the time of rise in titre of each patient was plotted against the time of the event. The main possibilities were: the start of peritoneal dialysis;

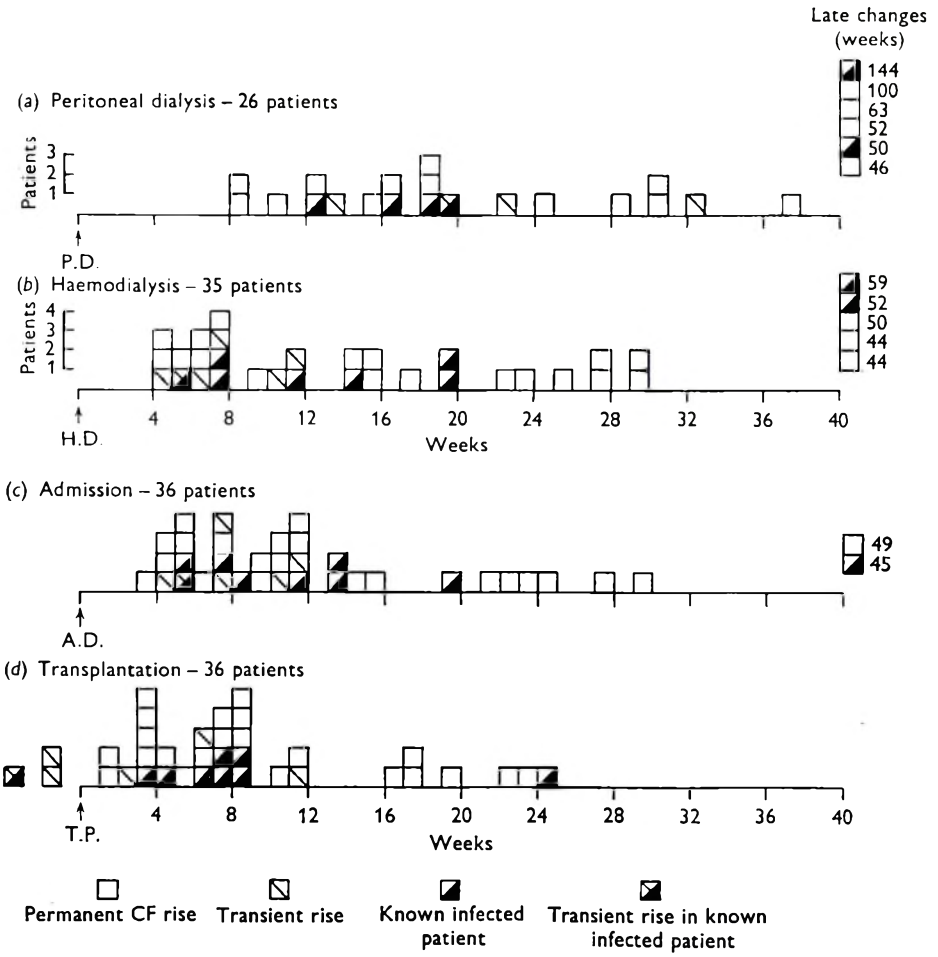

Fig. 3. Time of rise of CF titre in relation to peritoneal dialysis, haemodialysis, admission to hospital and first transplant.

haemodialysis; admission to hospital, and transplantation (Fig. 3*a–d*, Table 5). Blood transfusion was more difficult to assess, it occurred after admission and in some instances records are incomplete because of transfusions before patients were transferred to Cambridge. Peritoneal dialysis often preceded haemodialysis by weeks or months and was the first procedure in 32/37 patients. The appearance of Fig. 3(*a*) does not suggest that it was related to antibody changes. Haemodialysis was succeeded by a rise in titre after 4 weeks, an interval common to a number of reports dealing not only with renal transplantation (Craighead, 1969)

but post-perfusion (Henle *et al.* 1970; Caul *et al.* 1971) and post-transfusion (Purcell *et al.* 1971). It was associated with an earlier grouping of rises but they still extended for a long period. The relationship to admission was more definite as the majority of changes occurred 2–3 months earlier. Admission to hospital exposes a patient to infection from other patients, to the start of haemodialysis and blood transfusions and often precedes transplantation by only a day or two. It clearly could involve several factors. Transplantation (Fig. 3*d*) was preceded by three rises and three followed within 3 weeks afterwards. Transplantation could

Table 5. *Time of rise of CMV CF titre in relation to start of four procedures*

Procedure	Number of patients	Number with titre rise (including transient rises)	Number with rise within 12 weeks	Range of times to rise in weeks
Peritoneal dialysis	37	26	3	9–144
Haemodialysis	47	35	16	5–59
Admission	50	36	23	3–49
Transplantation	50	36	29	<4–25

Table 6. *Time between admission and transplantation, and start of haemodialysis and transplantation, arranged according to number of weeks after transplantation to rise of CF titre*

Weeks to CF titre rise after transplant	Weeks between admission and transplant			Weeks between start of haemodialysis and transplant		
	Patients	Average time	Range	Patients	Average time	Range
0–4	8	3·5	1–6	8	19	1–52
5–8	10	3·7	0–12	9	15	1–52
9–12	7	2·8	0–5	7	10·5	0–52
13–16	1	7	.	1	9	.
17–20	4	7	4–10	4	8	4–10
21–	3	8	0–20	3	18	4–27

not be the cause of the first three and may not have been the cause of all the second three changes as they are earlier than is usual. The distribution of rises in general was less extended than after admission so that transplantation appears to be a significant factor.

All three rises before transplantation were transient i.e. of less than 3 months duration, in patients with no detectable CF antibody and the titres produced were only 1/4 (2) and 1/8 (they are discussed further in the paragraph on patients without CF antibody). All had haemodialysis and transfusions in the weeks preceding their rises and these may have been the cause.

The three patients with rises within 3 weeks after transplantation all had IgG initially and two had a CF titre of 1/4. They produced CF rises to 1/64 (transient), 1/256 and 1/1028, with IgM also in the first and last, so they were similar to the majority of responses seen and may have been just slightly earlier than usual.

As haemodialysis preceded transplantation in this group of early risers the effect of haemodialysis on the whole group of patients was compared with the effect of admission (Table 6). It was found that the average time haemodialysis began before transplantation was 4 weeks later for each successive month that rises occurred in for the first 3 months after transplantation and the range of times before haemodialysis began was 0–52 weeks. This is not what would be expected if haemodialysis was a frequent precipitating factor.

The time from admission to transplantation was much more constant and the range less so that it was felt that the cause of the rises was related to admission.

This left admission and transplantation as the most important factors. Admission could lead to infection by cross-infection and by blood transfusions. Transplantation could lead to infection from the implanted kidney and from reactivation by the immunosuppressive treatment.

IgM antibody production

Twenty-one of the 50 patients produced CMV-specific IgM antibody, 20 after transplantation and one, who had a trace on admission, in increased amount. Nineteen of them produced IgG at the same time, either for the first time or in increased quantity, and in 12 the increase in titre of the two antibodies coincided. The relationship of IgM production to the initial CF titre is shown in Table 7.

Table 7. *IgM production and initial CF titre*

	Initial CF titre (reciprocal)										
	0	2	4	8	16	32	64	128	256	512	Total
Number of patients	10	7	11	6	5	6	2	1	1	1	50
IgM produced	6	3	2	3	1	4	1	0	0	1	21
IgM + CF rise	6 (2)	3	2 (1)	3	1	3	1	0	0	0	19 (3)
Total number with CF rise	10 (4)	4	8 (1)	4 (1)	4	4	2	1	0	0	37 (6)

( ) = proportion of patients with transient rise in titre.

When the patients were divided into two groups with highest initial CF titres in one and lowest in the other there was no difference in frequency of IgM production. A patient with a low or absent initial CF titre was therefore no more or less likely to produce IgM than a patient with a high initial CF titre.

Half of the patients with transient CF rises produced IgM so that a transient CF rise may be as significant as a longer lasting one.

The production of IgM in relation to the time of CF rise after admission and transplantation is shown by Fig. 4 to be evenly distributed. The duration of IgM production appeared to be prolonged and was frequently found to last for 2 years or more (Table 8), the longest duration recorded so far is 136 weeks and cessation of production has not yet been noted.

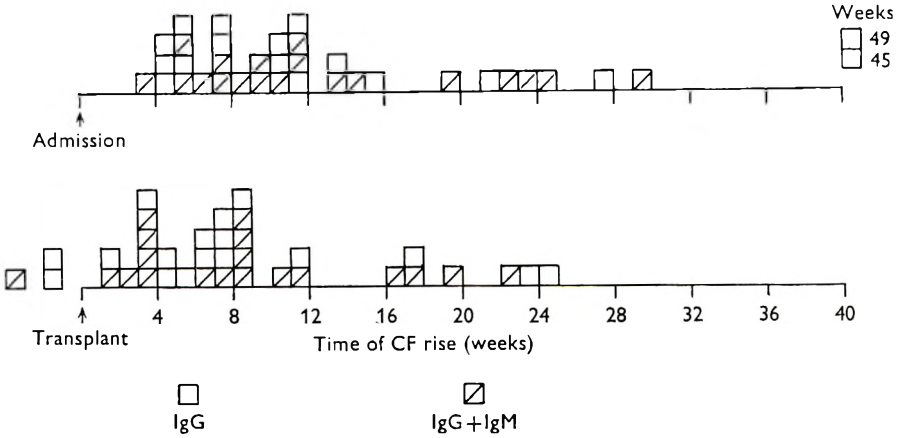

Fig. 4. IgM producers in patients with CF antibody rise, relationship to times of admission and transplantation.

Table 8. *Duration of IgM production*

	Observations still in progress	Patient died or transferred
0–1 year	7	5
1–2 years	3	3
2–3 years	3	0

Table 9. *Comparison of titre changes in 15 infected patients with the rest*

CF and IgG titres	Known infected patients IgM		Remainder of patients IgM	
	Positive	Negative	Positive	Negative
No change	1	3	1	6
Transient rise	1	0	3	2
Permanent rise	4	5	11	11
Fall	0	1	0	1
Total	6	9	15	20

Infected patients

To see if IgM production was mainly found in those patients with evidence of infection by virus isolation or histology these were compared with the rest of the patients (Table 9).

Fifteen patients were found to be infected; nine by examination of 15 post-mortems; one at post-mortem and own kidney at nephrectomy 3 weeks earlier; two from allografts removed for rejection (4 and 24 weeks after transplantation) and three nephrectomy specimens of the patients' own kidneys. The kidney of one of these was removed 11 weeks *before* transplantation.

The absence of IgM did not mean the absence of infection because it was not found in nine of the 15 infected patients.

In the various serological categories in Table 9, 35 patients were not known to be infected, mainly because specimens were not examined. The proportion of these with IgM, 15/35, is similar to the proportion in the known infected patients. The absence of any clear serological difference between the two groups leads to the obvious suggestion that they are both the same and therefore all, or most, of those not known to be were in fact infected.

If a fourfold or greater rise in titre is taken to indicate infection a total of 43/50 (86 %) were infected. This figure is derived from 37 patients with CF titre increases; two patients with IgM production only, that were positive *post mortem*; three post-mortem positives with no change in CF titre (two at 1/2 and one at 1/4) and no IgM production; and one post-mortem positive with a fall in CF titre from 1/32 initially, with no IgM production. This is comparable to a similar group of 43 patients examined in Denmark by Andersen & Spencer (1969) who found 33/36 (91 %) infected.

Patients with no detectable CF antibody in their initial sera

The 10 patients without CMV CF antibody were of particular interest for the interpretation of the significance of negative CF results.

Table 10. *Initial CF-negative patients*

			Initial	Subsequent antibody production					
	Patient	Age	IgG	IgM	IgG	Max CF	Onset of CF rise (wks)		
1.	A.C.	20	–	+	+	512	11	Post-T.P.	.
	D.T.	38	(tr)	+	+	32	9	Post-T.P.	Widespread infection p.m.
	A.M.	18	(tr)	+	+	1024	2	Post-T.P.	.
	P.Sp.	33	+	+	+	128	4	Post-T.P.	.
2.	D.B.	22	+	+	+	4	8	Post-T.P.	Terminal serum
	M.McW	31	–	–	+	4	5	Post-T.P.	Terminal serum
	R.S.	22	–	–	+	4	7	Post-T.P.	Prolonged CF titre
	M.O'M	33	+	+*	+	4	4	Pre-T.P.	Widespread infection p.m.
	J.R.	41	+	–	+	8	2	Pre-T.P.	.
	M.S.	31	–	–	+	8	3	Pre-T.P.	.

* 30 weeks after CF rise.

From Table 10 it can be seen that they fall into two groups according to their subsequent antibody production.

Group 1. Four patients produced appreciable CF antibody, after transplantation in each instance, and three of the four had initially at least a trace of CMV IgG by the FA method. The most probable interpretation seems to be that this antibody was significant, that infection was therefore not primary, and consequently IgG production could occur without inhibition by the immunosuppressive treatment. The time of antibody rise was similar to that of patients with initial CF antibody, as Purcell *et al.* (1971) found.

Alternatively, if they are considered primary infections, the FA IgG must be disregarded and the assumption made that immunosuppression did not inhibit IgG production.

Group 2. The remaining six patients failed to produce appreciable CF antibody. In two (D.B., M.McW) perhaps because the sera were collected within a month of death, a further rise might have occurred had they survived; in one the trace of CF antibody was maintained for months afterwards, but in the other three all the rises were pre-transplantation and transient.

One of these (M.O'M.) died with widespread CMV infection and could have been a primary infection, unable to produce more IgG because of immunosuppression, although she produced some IgG as judged by FA, before and after transplantation. Her antibody titres are shown in Fig. 5.

No post-mortem specimens were available from the other patient who produced IgM (D.B.). He may also have been a primary infection.

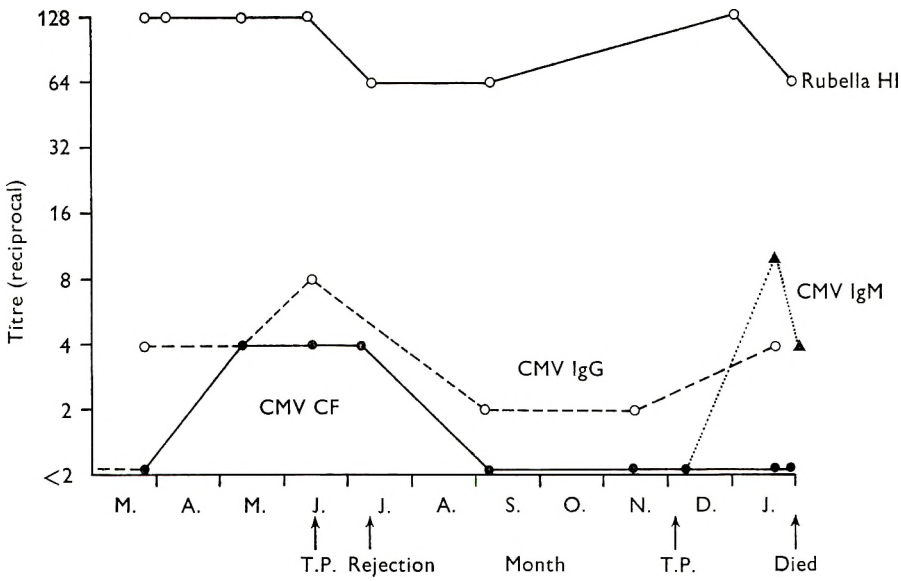

Fig. 5. Antibody production in patient M.O'M.

DISCUSSION AND CONCLUSIONS

In the work described the FA method was a useful supplement to CF and together they provided evidence that the incidence of previous CMV infection was at least 84% and possibly up to 92%. The antibody production by some CF-negative patients with no IgG or only a doubtful trace by immunofluorescence suggested that they may also have been previously infected so that the incidence of infection before transplantation was probably higher still.

The absence of CF antibody is inadequate evidence that previous CMV infection has not occurred which Weller (1970) has emphasized. The technique is not highly sensitive and the neutralization test is probably better. It may well be significant that wherever similar groups are examined before surgery the maximum incidence of CF-positives, which occurs usually in the group aged 30 years and over, is in the region of 80–86%. In Cambridge we found 84%, in Boston there

were 86% (Craighead, 1969), in Washington 81% (Rowe *et al.* 1956) and in Bristol there were 86% (Caul *et al.* 1971). When younger patients are included in the groups the total is again similar – Cambridge 66%, Boston 65%, Bristol 64%, in blood donors in Rumania 64% (Diosi, Moldovan & Tomescu, 1969) and in Finland 66% (Klemola, von Essen, Paloheimo & Furuhjelm, 1969).

It is known that neutralizing antibody is more common than CF antibody in the normal population (Carlstrom, 1965) and that the CF antibody titre can fall to undetectable levels in some children 2 or 3 years after infection (Starr, Calafiore & Casey, 1967; Lang & Noren, 1968; Andersen, 1969) so it seems highly probable that at least some of the 14% or so of adults without CF antibody are individuals in whom infection has occurred and the CF titre has subsequently fallen. This is supported by Andersen & Spencer (1969) who found neutralizing antibody in seven of their 10 renal transplant patients who had no CF antibody in their initial sera. The figure of 86% may represent a population maximum for the CF method.

The ability of the renal transplant patients to produce IgG was not greatly affected by immunosuppressive therapy when they had had their primary infection before immunosuppression started, as was the case with rubella, and this result is in agreement with the work of Rowley, Mackay & McKenzie (1969) who examined the immune response to a bacterial antigen in Melbourne patients on a comparable immunosuppressive regime to ours.

The production of CMV IgG was not greatly affected by immunosuppression in most patients, which is what would be expected. Immunosuppression was a possible reason for the absence of production in some of the patients with no detectable initial CF antibody who may have been primary infections or may have had practically no residual capacity for CMV IgG production.

The production of CMV IgG was as common in the patients not known to be infected as it was in those found to be infected from culture or histology of nephrectomy and post-mortem specimens, which suggested that they were equally infected. Not all of the infected patients produced IgM, perhaps because there was insufficient antigenic stimulus, perhaps related to circulating IgG. However, when IgM was produced it continued to be formed steadily for long periods, perhaps for as long as the patient survived because none have so far become negative and the longest recorded period of synthesis is 136 weeks.

This is also compatible with the observations of Rowley *et al.* (1969) who found that repeated antigenic stimuli under immunosuppression could cause primary type responses with IgM predominating, because this immunosuppressive technique is less effective for the inhibition of IgM than it is against IgG. Lang & Hanshaw (1969) cite Uhr & Finkelstein (1967) for the demonstration that repeated small doses of antigen can produce an IgM response alone in patients not on immunosuppression.

Thus IgM production implies constant or repeated production of antigen and therefore active infection.

In patients that are not immunosuppressed CMV IgM production is taken as evidence of primary infection, especially when there are no demonstrable initial

antibodies (e.g. Lang & Hanshaw, 1969; Caul *et al.* 1971) and in immunosuppressed patients evidence of primary infection was suggested from the observations of Craighead *et al.* (1967).

It is important to stress that in this group of immunosuppressed patients IgM synthesis does only mean active infection. It cannot be taken further and considered evidence for primary infection because nearly all have evidence of having had their primary infection in the past.

It is not possible to say that any were unequivocal primary infections in this series. There were no patients without CMV antibody initially who then produced IgM during immunosuppression, but little or no IgG (patients D.B. and M.O'M. in Table 10 are the nearest, with four others as possibles, M.McW., R.S., J.R. and M.S.).

Herpesviruses are known to be capable of reactivation, especially during immunosuppression (Rifkind, 1966; Kanich & Craighead, 1966; Montgomerie *et al.* 1969) and this could explain the active CMV infections seen in this group without the need for re-infection to have occurred (Carlstrom, 1965; Anderson & Spencer, 1969). Kanich & Craighead (1966) did not find evidence of CMV infection in those renal transplant patients not given immunosuppressives.

However, immunosuppressed patients are rendered susceptible to fresh infection as is only too well known and their environment contains infective CMV (Craighead, 1969) as well as do many of the kidneys transplanted and at least 5% of units of fresh blood they may be given (Diosi *et al.* 1969; Henle *et al.* 1970; Klemola *et al.* 1969). The situation is more complex than post-perfusion for open-heart surgery where primary infections have been described (Henle *et al.* 1970; Lang & Hanshaw, 1969; Paloheimo *et al.* 1968) yet even here reactivation is thought to be a factor by some (Klemola *et al.* 1969; Caul *et al.* 1971) whilst others maintain reactivation is the main factor (Purcell *et al.* 1971).

To resolve this argument detailed comparison is required between the primary infecting CMV strain and virus recovered subsequently. There are likely to be a range of antigenic differences between strains (Weller, Hanshaw & Scott, 1960; Dreesman & Benyesh-Melnick, 1967; Krech & Jung, 1969) but it does appear from the preceding observations that reactivation could be a major factor in the series described.

I am indebted to Miss M. F. Lawrence for technical assistance.

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Effects of oxygen on aerosol survival of radiation sensitive and resistant strains of *Escherichia coli* B

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SUMMARY

The aerosol survivals in air and nitrogen of radiation sensitive and resistant mutants of *Escherichia coli* B have been determined with logarithmic and resting phase bacteria. No consistent correlation was found between radiation sensitivity and aerosol sensitivity in the strains tested. Hence, the phenotypes Fil Hcr Exr, which determine sensitivity to radiation, do not influence aerosol survival, i.e. these known mechanisms which repair radiation-induced damage do not operate in aerosol stressed *E. coli*. In all cases the survival in air was less than that in nitrogen particularly so for *E. coli* B_{s-1}. The effect is explained in terms of a toxic action of oxygen. Comparison of survival of log and resting phase bacteria show that log phase cells are less aerosol stable than are resting phase cells. The ability to synthesize DNA in bacteria collected from the aerosol was less than in control unstressed bacteria, and this effect was independent of the presence of oxygen. Reduced ability to synthesize DNA could have been caused by reduced metabolic activity. It is shown that two different death mechanisms occur simultaneously in aerosols at low relative humidity. One mechanism is oxygen dependent and the other oxygen independent. The former was not through a decrease in metabolic activity, whereas the latter could be.

INTRODUCTION

Aerosol survival of micro-organisms has been reviewed in general terms by Anderson & Cox (1967). For bacteria there appears to be at least two different death mechanisms. There is a toxic action of oxygen (Ferry, Brown & Damon, 1958; Hess, 1965; Cox, 1966*a*, 1968*b*, 1970, 1971; Cox & Baldwin, 1966, 1967; Benbough, 1967, 1969; Webb, 1967, 1969) the effect of which occurs below 70% relative humidity (RH) (Cox, 1966*a*, 1968*b*; Benbough, 1967, 1969), although Webb (1967, 1969) did not observe the effect above 40% RH. Cox & Heckley (1972) have described a kinetic model which accounts for the toxic action of oxygen and indicates that the normally detected free radicals are not involved in oxygen toxicity. The other death mechanism is owing to dehydration and rehydration (Cox, 1965, 1966*a*, *b*, 1967, 1968*a*, *b*, 1969, 1970, 1971; Cox & Baldwin, 1966; Hatch &

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Dimmick, 1965, 1966; Hatch & Warren, 1969; Hatch & Wolochow, 1969; Hatch, Wright & Bailey, 1970). However, Webb (1965, 1967, 1969) does not consider that rehydration is important with regard to aerosol survival. It is possible for the two death mechanisms to occur simultaneously, especially at low RH (Cox & Heckley, 1972).

Recently Webb (1969) has suggested that mechanisms exist which repair damage caused by dehydration in the aerosol and implies that these mechanisms are those involved in repair of UV damaged DNA. However, Webb (1969) only considered excision repair. The present paper investigates excision repair, filament formation and the mechanism involved in repair of damage caused by X-rays. The approach was to measure the aerosol survival in air and nitrogen of five radiation resistant and sensitive mutants derived from *Escherichia coli* B. Also, the net DNA synthesizing ability, and the metabolic activity, of some of these mutants was measured after the bacteria were stressed in the aerosol and collected.

MATERIALS AND METHODS

Organisms

The *Escherichia coli* strains used in this study are listed below.

Strain	Phenotype	Derived from	Source	UV Sensitivity
B/r (Hill)	$Fil^-Hcr^+Exr^+$	B	Hill	Resistant
B/r (Witkin)	$Fil^-Hcr^+Exr^+$	B	Witkin	Resistant
B_{s-1}	$Fil^+Hcr^-Exr^-$	B	Hill	Sensitive
26x	$Fil^-Hcr^-Exr^-Thr^-$	B_{s-1}	Witkin	Sensitive
26xA3	$Fil^-Hcr^+Exr^+Thr^-Pro^-$	26xA2*	Witkin	Resistant

* 26xA2 ($Fil^-Hcr^-Exr^+$) derived from 26x.

The convention for the different phenotypes is given below.

Phenotype	Characteristic
Fil	Filament formation, etc.
Hcr	Host cell reactivation and excision repair capacity.
Exr	Sensitivity to X-ray damage.

Growth of organisms

(i) *Resting phase.* The bacteria were grown in a tryptone medium (Cox, 1966*a*) at 37° C. with shaking. After 18 hr. growth the bacteria were harvested by centrifugation and were resuspended in double-glass-distilled water. This suspension was used to generate the aerosols.

(ii) *Log phase.* The bacteria were grown in nutrient broth, as for the resting phase bacteria. After 18 hr., 0·1 ml. of this culture was used to inoculate a fresh batch of nutrient broth (100 ml.). After 4 hr. growth at 37° C., with shaking, the bacteria were harvested by centrifugation and resuspended in fresh nutrient broth. This culture was used to generate the aerosols.

Aerosol apparatus

A rotating drum apparatus was used, as described by Cox (1966*a*). Aerosol samples were collected by impingers, as described by Cox (1966*a*). Usually the experimental conditions were 20 % RH and 26·8° C.

Assay

Suspension and aerosol samples were diluted as required and plated on nutrient agar. After overnight growth at 37° C., the colonies were counted.

Tracer

Bacillus subtilis var. *niger* spores were used as a tracer (Cox, 1966*a*; Anderson & Cox, 1967). The ratio of *Escherichia coli* to spores was calculated for the suspension and for the aerosol samples. Viabilities were taken as the ratio of coli to spore count, normalized to the ratio in the spray suspension as being 100 % survival. The latter was measured before and after spraying.

Net DNA synthesis

For these experiments the strains of *Escherichia coli* were grown in a lactate medium composed of 0·004 g. P as orthophosphate, 10 g. sodium lactate, 5·2 g. NaCl, 1·8 g. K_2SO_4, 0·12 g. $MgSO_4$, 1·0 g. NH_4Cl, 0·5 g. Bactopeptone, per 1000 ml. distilled water. The pH was 6·8. The suspension used to produce aerosols was then prepared as for resting phase cultures, except that the spores were omitted.

Since larger populations than are normally collected from aerosols were required for the determination of net DNA synthesis, suspensions containing 10^{11} bacteria/ml. were disseminated with a 3-jet Collison spray and aerosol samples (30 min. aerosol age) were collected for 2·5 min. in an impinger. The optical density of the collected aerosol (determined by a Beckman spectrophotometer) was matched by that of a control, obtained by diluting the suspension used for generating the aerosol. In all cases the bacteria in the aerosol sample and in the control were suspended in the lactate medium plus deoxyadenosine (250 μg/ml.) at a density of 1·1 to $1{\cdot}7 \times 10^8$ bacteria/ml. The two suspensions were rapidly warmed to 37° C. and at t = 0, 0·3 ml. [^{3}H]thymidine was added to each suspension at 37° C.; the suspensions then contained 38 μg/ml. and 1·2 μCi/ml. of [^{3}H]thymidine. At intervals (the shortest being 30 sec.) 0·2 ml. was removed from each suspension and pipetted onto separate Millipore filters (25 mm., 0·22 μm.) previously wetted with ice-cold 1 N-NaOH. After filtration the bacteria were washed with a total of 15 ml. of ice-cold 5 % (w/v) trichloroacetic acid (TCA), and then with absolute ethanol. After drying, the bacterial samples on the Millipore filters were put into separate 10 ml. volumes of scintillation fluid ('Permablend' from Packard Instruments). The amount of [^{3}H]thymidine incorporated into the cold TCA insoluble material was determined in a Packard Scintillation Spectrometer (Model 4322).

Oxygen uptake

The bacteria were grown, aerosolized and collected, as for the experiments to measure net DNA synthesis, except that the collecting fluid was phosphate buffer (Cox, 1966*a*). A suspension containing 2×10^8 bacteria/ml. was put in the side-arm of a Warburg flask, KOH was in the centre well, and the lactate medium was in the main compartment of the flask. The metabolic activity of bacteria collected from the aerosol, and of an unstressed control, was determined by the Warburg technique, at 37° C.

All survival, net DNA synthesis and metabolic activity experiments were performed between two and five times.

RESULTS

Aerosol survival of Escherichia coli *B/r*

Fig. 1 shows the aerosol survival of *Escherichia coli* B/r (Hill) at 20% RH (26·8° C.) for log and resting phase bacteria in air and nitrogen (> 99·997%)

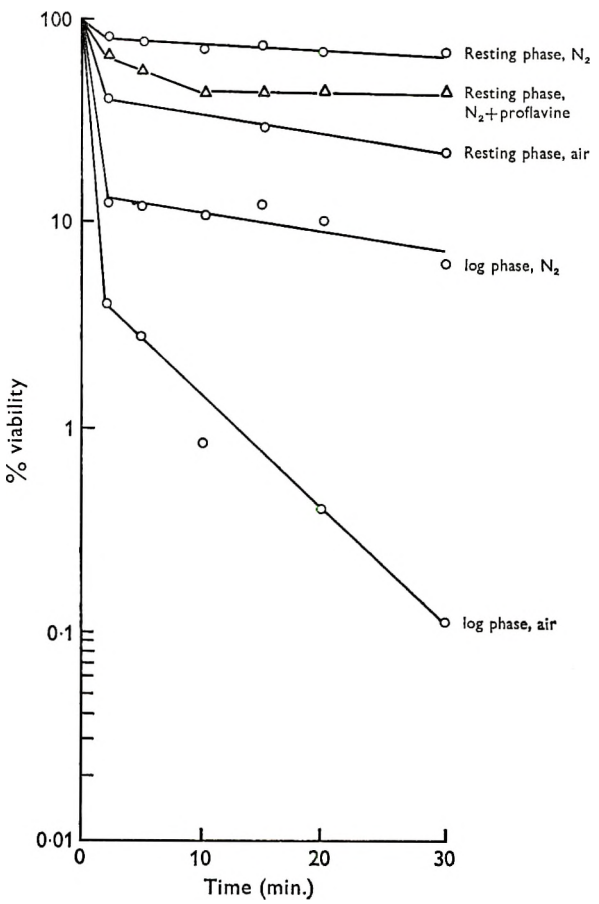

Fig. 1. Aerosol survival of *Escherichia coli* B/r (Hill) at 20% relative humidity and 26·8° C

(Matheson Company). Also included are results obtained when the collected aerosol samples were plated onto nutrient agar plus proflavine (5 μg./ml.) (an excision repair inhibitor, Witkin, 1963; Lieb, 1964). The results show that survival of resting phase and of log phase bacteria was greater in nitrogen than in air. Under comparable conditions, resting phase bacteria were more aerosol stable than were log phase bacteria. The addition of proflavine to the nutrient agar had only a slight inhibitory effect.

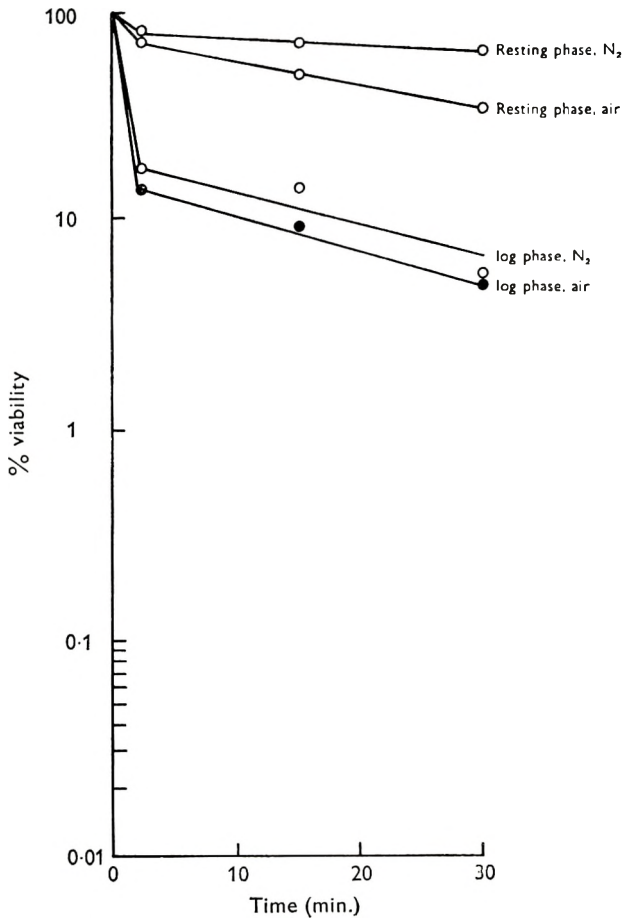

Fig. 2. Aerosol survival of *Escherichia coli* B/r (Witkin) at 20 % relative humidity and 26·8° C.

Fig. 2 is similar to Fig. 1 except that the Witkin strain of *Escherichia coli* B/r was used. The results suggest that the Witkin strain is a little more resistant to aerosol damage than is the strain of Hill, especially for log phase bacteria in air. Again only a slight inhibitory effect of the addition of proflavine (5 μ./ml.) to the nutrient agar was found, as for Fig. 1.

Aerosol survival of Escherichia coli B_{s-1}

The data of Fig. 3 were obtained under the same conditions as for Figs. 1 and 2, but using the B_{s-1} strain. As with the B/r strains, survival in air was less than in

nitrogen, although with strain B_{s-1} the difference was much greater. Log phase bacteria were again more unstable than resting phase bacteria, for comparable conditions.

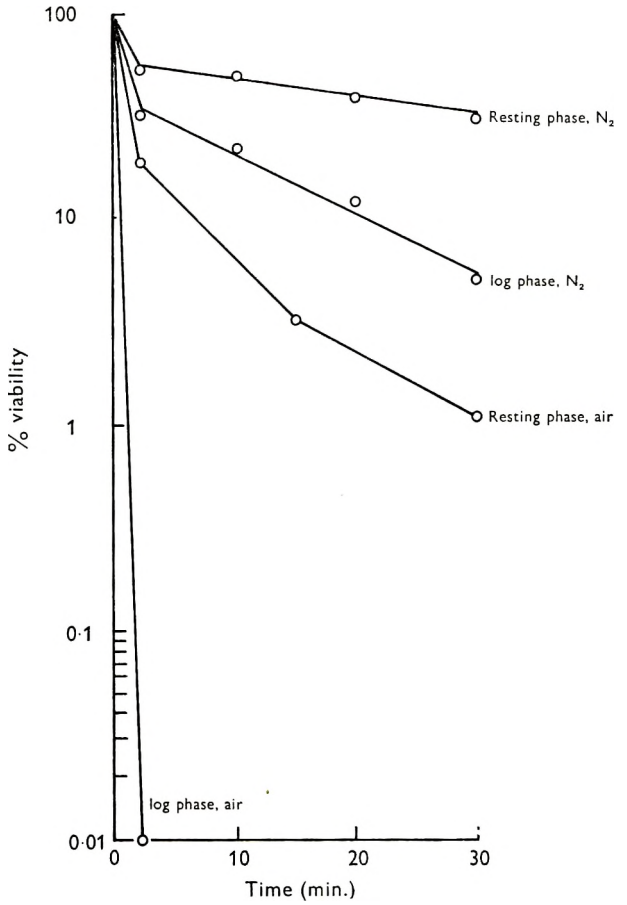

Fig. 3. Aerosol survival of *Escherichia coli* B_{s-1} at 20 % relative humidity and 26·8° C.

Aerosol survival of Escherichia coli 26x

As with the other strains, air is toxic compared with nitrogen (Fig. 4). The actual survival under the different conditions was similar to that of strain B/r (Figs. 1 and 2).

Aerosol survival of Escherichia coli 26xA3

As with *Escherichia coli* 26x (Fig. 4) the results (Fig. 5) for *E. coli* 26xA3 are similar to those of strain B/r (Figs. 1 and 2), except for resting phase bacteria in air, which resembled strain B_{s-1} behaviour.

Net DNA synthesis in aerosol-stressed Escherichia coli *B/r*

Net DNA synthesis in aerosol-stressed *Escherichia coli* B/r was less than that in the unstressed bacteria (Fig. 6). However, the inhibition was the same for

samples collected from aerosols stored in nitrogen and air, i.e. the presence of oxygen did not have any effect on net DNA synthesis of stressed *E. coli* B/r. The 30 min. viabilities at 36 % RH and 26·8° C., were 24 % in nitrogen and 2·1 % in air.

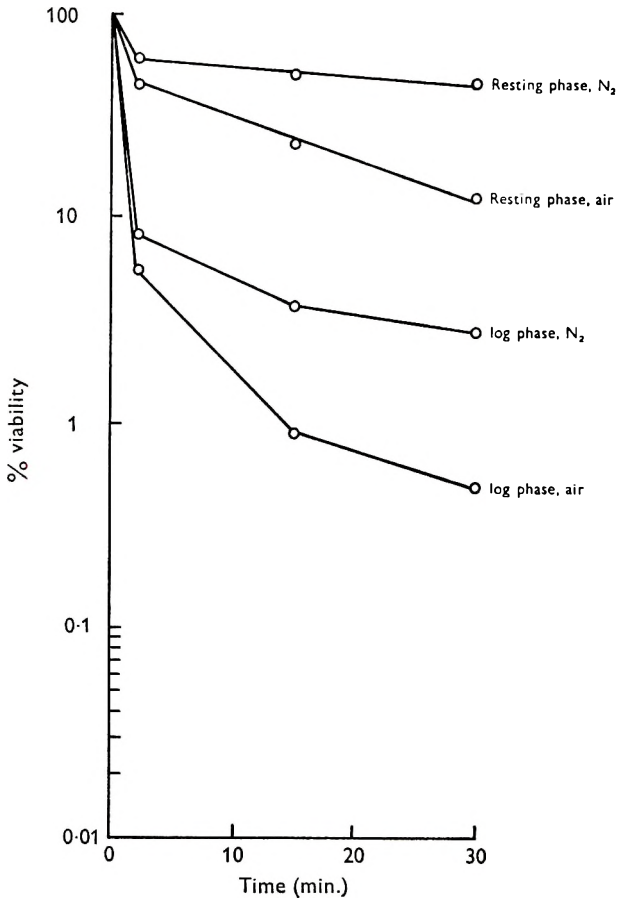

Fig. 4. Aerosol survival of *Escherichia coli* 26x at 20 % relative humidity and 26·8° C.

Net DNA synthesis in aerosol-stressed Escherichia coli B_{s-1}

The ability of aerosol-stressed *Escherichia coli* B_{s-1} to synthesize DNA was inhibited compared with unstressed controls (Fig. 7). The result obtained was similar to that with *E. coli* B/r (Fig. 6) even though the 30 min. viabilities at 36 % RH and 26·8° C. of strain B_{s-1} were 13 % in nitrogen and 0·014 % in air. Again, oxygen was not involved in causing inhibition of net DNA synthesis (Fig. 7).

The kinetics of [^{3}H]thymidine incorporation in *Escherichia coli* B/r (Fig. 6) and *E. coli* B_{s-1} (Fig. 7) were not exactly the same, especially at 30 sec.; the reason for this difference is not known, but is not an artifact resulting from insufficient washing.

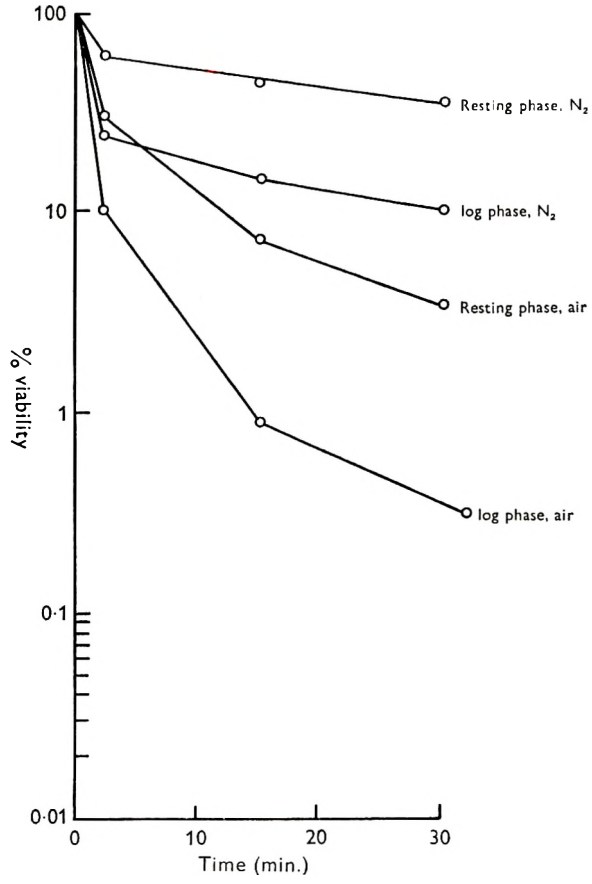

Fig. 5. Aerosol survival of *Escherichia coli* 26xA3 at 20% relative humidity and 26·8° C.

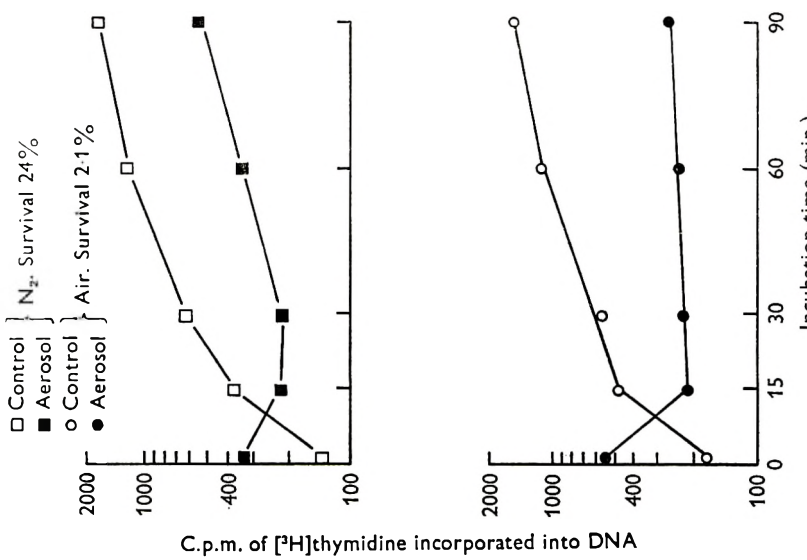

Fig. 6. Net DNA synthesis in *Escherichia coli* B/r (Hill) collected from an aerosol stored at 36% relative humidity and 26·8° C. 30 min. survival in nitrogen 24% and in air 2·1%.

Oxygen uptake by aerosol-stressed Escherichia coli

The data of Figs. 8 and 9 show that aerosol-stressed *Escherichia coli* B/r and B_{s-1} have lower metabolic activity than unstressed control bacteria. The degree of reduction in metabolic activity was independent of the presence of oxygen in the aerosol phase.

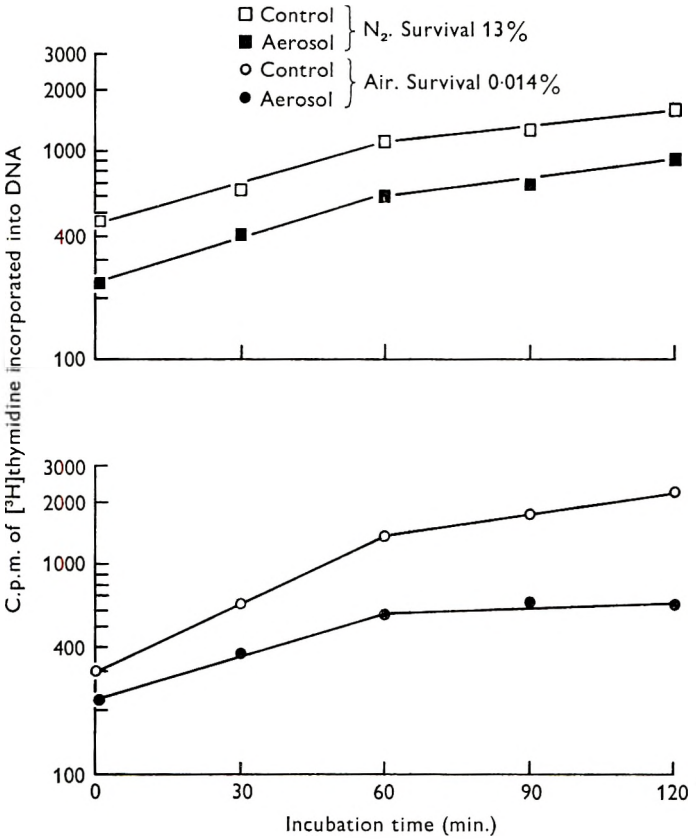

Fig. 7. Net DNA synthesis in *Escherichia coli* B_{s-1} collected from an aerosol stored at 36% relative humidity and 26·8° C. 30 min. survival in nitrogen 13% and in air 0·014%.

DISCUSSION

Aerosol survival data are summarized in Table 1, which gives the 30 min. survival values of the different strains (resting and log phase) in air and nitrogen, at 20% RH and 26·8° C.

As seen from Table 1 and Figs. 1–5, there does not appear to be an overall correlation between aerosol survival in air, or in nitrogen, and the Hcr, Exr phenotypes of the six strains of *Escherichia coli*. For log phase bacteria in air there may be a correlation with Fil, since *E. coli* B and B_{s-1} are both Fil^+ and in air both are much less stable than the Fil^- strains, i.e. Fil^+ for these conditions could be involved in oxygen-induced damage. However, preliminary data show log phase *E. coli* B to be more unstable in nitrogen than is *E. coli* B_{s-1} (Table 1).

When decay in air is corrected for the decay in nitrogen, the toxic action of oxygen is much greater for *E. coli* B_{s-1} than for *E. coli* B, i.e. the apparent correlation with Fil$^+$ may be an artifact. Further work is required to substantiate the possible role of Fil.

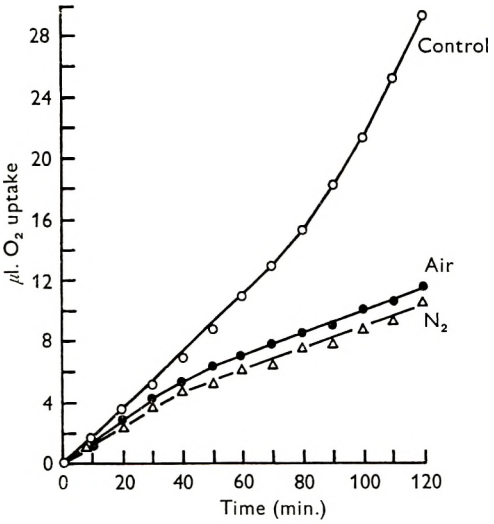

Fig. 8

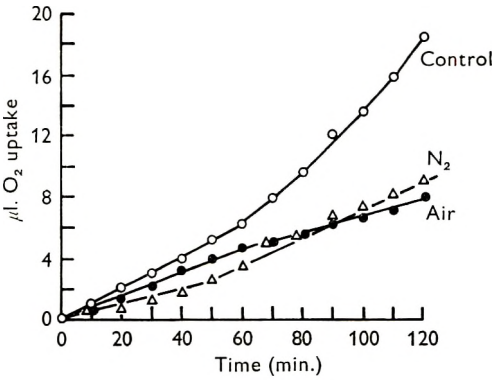

Fig. 9

Fig. 8. Oxygen uptake by *Escherichia coli* B/r collected from aerosols in air (●) and in nitrogen (△). (○) Control.

Fig. 9. Oxygen uptake by *Escherichia coli* B_{s-1} collected from aerosols in air (●) and in nitrogen (△). (○) Control.

Table 1. *Survival values* (30 *min.*) *of strains of* Escherichia coli *stored at* 20 % *relative humidity* (26·8° *C.*)

		Survival % log phase		Survival % resting phase	
Strain	Phenotype	Air	Nitrogen	Air	Nitrogen
B/r (Hill)	Fil$^-$Hcr$^+$Exr$^+$	0·1	7	21	75
B/r (Witkin)	Fil$^-$Hcr$^+$Exr$^+$	5	7	35	65
B*	Fil$^+$Hcr$^+$Exr$^+$	0†	0·3	1	76
B_{s-1}	Fil$^+$Hcr$^-$Exr$^-$	0‡	5	1	30
26x	Fil$^-$Hcr$^-$Exr$^-$	0·5	3	12	44
26xA3	Fil$^-$Hcr$^+$Exr$^+$	0·3	10	3	35

* Cox, unpublished preliminary data.
† 2 min. value 1·2 %.
‡ 2 min. value 0·01 %.

In air, survival was always lower than in nitrogen, although the degree of difference depended upon the strain of *Escherichia coli*. These results may be explained in terms of a toxic action of oxygen (Ferry, Brown & Damon, 1958; Hess, 1965; Cox, 1966*a*, 1968*b*, 1970, 1971; Cox & Baldwin, 1966, 1967; Cox &

Heckly, 1972; Benbough, 1967, 1969; Webb, 1967, 1969). Even though the presence of oxygen decreased survival, this decrease was not due to the influence of oxygen on net DNA synthesis or metabolic activity (Figs. 6, 7, 8 and 9; Benbough, 1967).

In nitrogen, strains of *Escherichia coli* are not completely stable, i.e. there is an oxygen-independent death mechanism (Figs. 1–5, Table 1; Cox, 1966*a*, 1968*a*, *b*, 1970, 1971; Cox & Baldwin, 1966, 1967; Benbough, 1967, 1969; Webb, 1967, 1969). This might be explained in terms of only partial repair to aerosol-induced DNA damage. However, the DNA repair mechanisms for radiation-induced DNA damage do not appear to operate for aerosol damage, because repair capability of radiation-induced DNA damage and aerosol survival are not related in nitrogen atmospheres (Figs. 1–5, Table 1). Also, the presence of proflavine (5 μg./ml.) in the plating medium did not greatly decrease the survival of two strains of *E. coli* B/r, even though proflavine inhibits host cell reactivation (Witkin, 1963; Lieb, 1964), i.e. excision repair. However, Webb (1969) reported a marked inhibition of *E. coli* B/r growth caused by the addition of proflavine (5 μg/ml.) to nutrient agar. The cause of this discrepancy is not known. Net DNA incorporation of [^{3}H]thymidine is partially inhibited in *Escherichia coli* B/r and *E. coli* B_{s-1} stored in air and nitrogen at 36% RH (Figs. 6 and 7). This reflects a partial inactivation of DNA synthesis, or an increased rate of DNA breakdown, which is independent of the presence of oxygen in the aerosol phase. The reduced metabolic activity of *E. coli* B/r and B_{s-1} shown in Figs. 8 and 9 is also independent of the presence of oxygen in the aerosol phase. Reduced metabolic activity could account for the reduced net DNA synthesis in these organisms. A determination of the physical integrity of DNA in bacteria collected from the aerosol might elucidate this question.

The data presented in this paper indicate that mechanisms involved in repair of radiation-induced damage (viz. Fil, Hcr, Exr phenotypes) do not operate for aerosol-induced damage. However, it is possible that other unknown mechanisms repair aerosol-induced dehydration-rehydration damage.

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The survival of salmonellas on finger-tips and transfer of the organisms to foods

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SUMMARY

The survival of salmonellas on the finger-tips is considered with reference to the ease with which they can be transferred to food by handling.

Escherichia coli and several *Salmonella* serotypes were shown to survive on the finger-tips for various periods of time, for example, *S. anatum* could be recovered 3 hr. after artificially contaminating them with between 500 and 2000 organisms. *S. anatum* could also be recovered from the finger-tips after contaminating them with more than 6000 organisms followed by a 15 sec. hand-wash 10 min. later. Similarly, the survivors from minimal inocula of less than 100 *S. anatum*/finger-tip were, after 10 min., still capable of infecting samples of corned beef and ham. *E. coli* was isolated from the finger-tips of 13 of 110 butchers soon after they had left the meat line at a meat products factory, but was not detected on the finger-tips of 100 volunteers at the Central Public Health Laboratory.

The implications of the present findings to the spread of salmonellas from raw to cooked foods, and the relevance of this to outbreaks of *Salmonella* infection in the general population and in hospitals, are discussed.

INTRODUCTION

It is not always clear how salmonellas of animal origin reach prepared foods and give rise to infection in man. Raw foods of animal origin, especially meat and poultry, are often contaminated with salmonellas, but outbreaks of infection may follow the consumption of adequately cooked foods. The circumstances of many outbreaks indicate that salmonellas may have been transferred from a raw to a cooked food by means of equipment, working surfaces or by the hands when both foods have been handled by the same person. There is still much emphasis on the spread of salmonellas from human faecal excretors to foods, but hitherto little attention has been paid to the ability of the human hand to transfer these organisms from one food to another. The aim of the present work was, therefore, to study the survival of salmonellas on the finger-tips and the ease with which they could be transferred to food by handling.

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MATERIALS AND METHODS

Volunteers

Preliminary tests to detect the presence of *Escherichia coli* on the finger-tips were made on 100 members of the staff at the Central Public Health Laboratory. These were 50 laboratory and 25 secretarial staff, and 25 persons who washed glassware usually wearing rubber gloves. In a similar experiment, the finger-tips of one hand of 110 butchers at a large meat products factory were sampled about 5 min. after they had left the meat line and before handwashing.

In experiments involving the artificial contamination of finger-tips with *E. coli* or salmonellas, tests were first made on the unwashed finger-tips of one or both of the authors. Similar experiments were then made on volunteers (9 male, 12 female) from the staff at the Central Public Health Laboratory. Volunteers did not use their contaminated hands during the period of each experiment, and after all the finger-tips had been sampled by the elution technique, the volunteers were instructed to wash their hands thoroughly with soap and water for at least 1 min. immediately, and again about 10 min. later.

Bacteria

The cultures used for the artificial contamination of finger-tips were *E. coli* (O-group 88), one culture each of *Salmonella anatum*, *S. derby*, *S. infantis*, *S. meleagridis* and *S. panama* that had been isolated during 1967–1968 from various foods of animal origin and stored on Dorset egg medium, and *S. senftenberg* 775W (N.C.T.C. 9959).

Media

MacConkey agar was used for the isolation of *E. coli* from the finger-tips in both the impression and elution methods described below.

Bismuth sulphite agar (Oxoid) and deoxycholate citrate lactose agar (Hynes, 1942) modified by the addition of 1 % sucrose, were used for the isolation of salmonellas from the contaminated finger-tips of volunteers. These media were also used for subcultures from liquid enrichment of corned beef and ham previously touched with contaminated fingers. In these experiments 25 ml. of lactose broth was used as an enrichment medium for *Salmonella* from corned beef, and selenite F medium (Leifson, 1936) modified by replacement of lactose with mannitol and sterilization by Seitz filtration, for recovery of *Salmonella* from ham. Different enrichment media were employed because corned beef is a sterile product and ham is not.

Diluent

The diluent used throughout the work was 0·045 M phosphate buffer containing 0·1 % peptone and 0·1 % Triton X-100 (a non-ionic detergent, Rohm and Hass Limited), at pH 7·9.

Finger impression plates for Escherichia coli

The finger-tips of one hand were gently pressed on the surface of a MacConkey agar plate for about 2 sec. The plates were incubated at 37° C. for 24 hr. and colonies morphologically resembling *E. coli* were picked for confirmation by biochemical tests.

Contamination of finger-tips with Escherichia coli *and salmonellas*

The tips of the fingers of volunteers were artificially contaminated at various times during the normal working day. Each finger-tip received one drop (0·0067 ml.) of a convenient dilution of an overnight broth culture of the test organism delivered from a no. 20 needle mounted on a syringe; the drop was spread over an area of about 0·5 cm.^{2} The number of organisms in the diluted culture was estimated by the method of Miles & Misra (1938).

Elution of organisms from finger-tips

To elute surviving organisms, the tip of each finger was rubbed against the bottom of a 2 oz. jar containing 1 ml. of diluent and then wiped on the rim to leave as much diluent in the jar as possible. Tenfold dilutions were prepared from 0·5 ml. samples of eluate and 0·02 ml. drops of each dilution plated onto selective media which were incubated at 37° C. for 48 hr. Colonies of *E. coli* or *Salmonella* were confirmed by biochemical or serological tests.

In experiments to determine the presence or absence of salmonellas, 1 ml. of warm (37° C.) double-strength lactose broth was added to the diluent in the sampling jars; the jars were incubated at 37° C. and plated after 24 and 72 hr. on bismuth sulphite and deoxycholate citrate sucrose agars.

Recovery of Escherichia coli *and salmonellas from the finger-tips after artificial contamination*

In a series of tests on different days, various inocula of *E. coli* were applied to the finger-tips of both authors; separate finger-tips were sampled after 2, 5, 10, 15 and 25 min. These experiments were repeated with 19 other volunteers with inocula in the same range, with sampling times of 5, 15, 30, 45 and 60 min.

Similar experiments were carried out with *S. anatum*, first on one of the authors, using a wide range of inocula, and then on eleven other volunteers with inocula ranging from 280 to 3500 organisms per finger-tip. The presence or absence of *S. anatum* in eluates from finger-tips, after contamination with low inocula, was also determined. In these tests various inocula from *ca.* 27 to 1800 were applied to the finger-tips of both hands of one of the authors; separate finger-tips were sampled at intervals up to 180 min.

The survival of different serotypes of *Salmonella* on finger-tips was also studied. One finger-tip on each hand of one of the authors was artificially contaminated with *S. anatum*, *S. derby*, *S. infantis*, *S. meleagridis*, *S. panama*, or *S. senftenberg* (twice). Inocula ranging from 3300 to 9500 organisms per finger-tip were used, and fingers were sampled 15 and 60 min. after contamination.

Effect of hand washing on the removal of salmonellas from contaminated finger-tips

In a series of tests on different days, various inocula of *S. anatum* from 830 to a million were applied to the finger-tips of one person. After 10 min. exposure, the hands were washed with soap and running warm water for 15 sec. in a standard manner and dried with individual paper towels. Each finger-tip was then rubbed against the bottom of an individual jar containing diluent, double-strength lactose broth was added and the jars incubated at 37° C. The experiments were repeated with seven other persons with inocula of *S. anatum* of *ca.* 250/finger-tip.

Artificial contamination of food with salmonellas from the finger-tips

Approximately 5 g. samples of corned beef from a freshly opened can were placed in 2 oz. jars. The finger-tips of 17 persons were artificially contaminated with inocula of *S. anatum* ranging from less than 10 to 6000. After 10 min. exposure, each finger-tip was pressed for about 5 sec. on an individual sample of corned beef. Twenty-five ml. of lactose broth were then added and the jars incubated at 37° C.; subcultures were made after 24 and 72 hr. on bismuth sulphite and deoxycholate citrate sucrose agars. The experiments were repeated on 14 persons using samples of ham; the inocula of *S. anatum* per finger-tip ranged from less than 10 to 330, and selenite F medium was used as the enrichment medium.

RESULTS

Although *E. coli* was not isolated from any of the finger-tips on the sampled hands of 100 of the staff at the Central Public Health Laboratory, it was detected on the finger-tips of the sampled hands of 13 of 110 butchers in the meat products factory.

Table 1 shows the percentage recovery of *E. coli* from the finger-tips after artificial contamination with various inocula. The percentage recovery was somewhat variable, but in general the range and median values obtained in replicate tests on the two authors were similar. After a contact time of 15 min. usually less than 1 % of the initial inoculum was recovered. Yet, in single tests on the finger-tips of nineteen other persons the percentage recoveries of *E. coli* were generally greater and for ten persons the initial inoculum was not reduced to 1 % until 60 min.

Table 2 shows the recovery of *S. anatum* from the finger-tips after artificial contamination with various inocula. In general, the percentage recoveries obtained from nine tests on one of the authors and one test on eleven other persons were similar. The percentage recovery of *S. anatum* fell sharply during the period 0–5 min. and then more slowly during the subsequent 55 min.

Table 3 shows the recovery of *S. anatum* from the finger-tips of one volunteer after contamination with inocula of different sizes. With the larger inocula, *S. anatum* was recovered at least 180 min. later, but with the smaller (less than 100 organisms per finger-tip) this time was reduced to 75 min. or less.

Table 4 shows the percentage recovery of various *Salmonella* serotypes from artificially contaminated finger-tips. The results show that after 15 or 60 min. the percentage recovery rates for *S. anatum*, *S. derby*, *S. infantis*, *S. meleagridis*, and *S. panama* were similar and all were greater than those for *S. senftenberg*.

Table 5 shows the effect of hand washing on the removal of *S. anatum* from contaminated finger-tips. The standardized hand washing procedure used did not

Table 1. *Recovery of* Escherichia coli *from artificially contaminated finger-tips*

No. of persons		1	1	19
No. of tests/person		7	13	1
Initial inoculum per finger-tip ($\times 10^4$)		15–180	3·5–180	32–46
Percentage recovery of *E. coli* after (min.)	2	82·2* (15–100)†	29·7 (11·2–96)	ND
	5	8·2 (0·46–13·6)	2·0 (0·27–9·0)	87·5 (6·2–100)
	10	0·70 (0·20–1·91)	1·1 (0·09–1·91)	ND
	15	0·42 (0·27–0·68)	0·36 (0·07–1·47)	13·8 (0·3–100)
	25	0·31 (0·03–1·05)	0·11 (0·03–0·68)	ND
	30	ND	ND	6·0 (0·02–100)
	45	ND	ND	8·7 (0·02–93·8)
	60	ND	ND	1·1 (0·02–40)

* Median value.
† Range (minimum–maximum).
ND = not done.

Table 2. *Recovery of* Salmonella anatum *from artificially contaminated finger-tips*

No. of persons		1	11
No. of tests/person		9	1
Initial inoculum per finger-tip ($\times 100$)		9·3–3900	2·8–35
Percentage recovery of *S. anatum* after (min.)	5	7·4* (2·36–14·7)†	4·4 (1·08–100)
	15	2·7 (0·43–10·7)	2·8 (1·08–18·3)
	30	1·1 (0·43–11·3)	2·4 (< 0·36–15·4)
	45	0·86 (0·21–8·3)	1·4 (< 0·36–3·9)
	60	0·40 (0·21–4·0)	0·72 (< 0·36–3·9)

* Median value.
† Range (minimum–maximum).

Table 3. *Recovery of* Salmonella anatum *from the contaminated finger-tips of a single volunteer*

Initial inoculum /finger-tip	Presence (+) or absence (−) of *S. anatum* in cultures from finger-tips after various time intervals (min.)											
	5	10	15	30	45	60	75	90	105	120	150	180
1800	ND	ND	+	+	+	+	+	+	+	+	+	+
530	ND	ND	+	+	+	+	+	+	+	+	+	+
80	+	+	+	+	+	+	+	−	−	−	ND	ND
36	ND	ND	+	−	−	−	−	+	−	−	−	−
27	+	+	+	−	+	−	−	−	−	−	ND	ND

ND = not done.

Table 4. *Recovery of salmonellas from finger-tips after contamination with different serotypes*

Salmonella serotype	Initial inoculum /finger-tip $\times 10^3$	% recovery of salmonellas from finger-tips after two time intervals (min.)	
		15	60
S. anatum	9·5	2·7	0·59
S. derby	3·4	3·3	1·8
S. infantis	3·3	3·3	1·1
S. meleagridis	7·6	3·9	1·7
S. panama	6·5	5·3	3·7
S. senftenberg 775W	6·6	0·24	0·18
	7·5	0·50	0·16

Table 5. *Recovery of* Salmonella anatum *from finger-tips after hand washing*

Initial inoculum/finger-tip	Proportion of finger-tips positive for *S. anatum*
1,000,000	10/10 (one person)
6,400	3/10 (one person)
830	0/10 (one person)
250	0/70 (seven persons)

Table 6. *Recovery of* Salmonella anatum *from corned beef* after contact with contaminated finger-tips*

Inoculum of *S. anatum*/ finger-tip	No. of finger-tips contaminated	No. of corned beef samples +ve for *S. anatum*	% of corned beef samples +ve for *S. anatum*
6000	60	60	100
600	40	40	100
60	40	36	90
7	25	4	16

* Plate count at 35° C. = <100 organisms/g. *Salmonella* not found in 50 g. − before contamination.

remove an inoculum of 10^6 *S. anatum*/finger-tip and all ten finger-tips remained contaminated. With inocula between 10^3 and 10^4 some finger-tips remained positive, but with inocula below 10^3 no salmonellas were grown after hand washing. The results from Table 2 indicate that a considerable reduction in numbers of salmonellas could be expected during the 10 min. period between contamination and hand washing.

Table 6 shows the recovery of *S. anatum* from corned beef after contact with contaminated finger-tips. All, or nearly all samples were contaminated from fingers with the three largest inocula, and only with the small inoculum of *ca.* 7 organisms/finger-tip did the positive samples fall to 16%. Similar results are shown with cooked ham in Table 7.

Table 7. *Recovery of* Salmonella anatum *from cooked ham* after contact with contaminated finger-tips*

Inoculum of *S. anatum*/ finger-tip	No. of finger-tips contaminated	No. of ham samples +ve for *S. anatum*	% of ham samples +ve for *S. anatum*
330	40	39	98
30	50	43	86
8	50	7	14

* Plate count at 35° C. = 3×10^5 organisms/g. *Salmonella* not found in 50 g. – before contamination.

DISCUSSION

The survival of bacteria on human skin depends on factors such as humidity, pH, the presence of antibacterial substances in the skin secretions and the presence of competitive organisms. In general, Gram-negative bacilli are more susceptible to desiccation than are Gram-positive cocci and most of them do not survive long on the skin (Ricketts, Squire & Topley, 1951; McDade & Hall, 1964). Payne (1949), for example, showed that the death rate of *E. coli* on skin depended on the rate of drying and was at a maximum between relative humidities of 40 and 50%. Coliform bacilli are not commonly found on normal skin although few workers have made an extensive or careful search for them. Williams & Miles (1949) reported the isolation of coliforms from 18 (3·6%) hands from 500 normal individuals. In contrast, Horwood & Minch (1951) using a hand-rinse technique reported that coliform bacilli were present on the hands of 22 (65%) of 34 food handlers; *E. coli* was found on 12 (38%) of the hands tested. In the present study *E. coli* was not isolated from the finger-tips of 100 of the staff at the Central Public Health Laboratory, but the organism was isolated from the finger-tips of 13 (11·8%) of the 110 butchers tested soon after leaving duty. The difference in the isolation rates of *E. coli* is probably attributable to the fact that the butchers' hands were continuously exposed to contamination from the meat they were handling. The isolation rate from the butchers would probably have been much higher if a finger-rinse technique had been used instead of the finger impression technique and if sampling had been done immediately after the butchers had left the meat line. We are unaware of any surveys on the frequency of isolation of

salmonellas from the hands, but as meat and poultry are important sources of these organisms occasions will arise when the finger-tips of butchers and food handlers are contaminated. Tables 6 and 7 show that cooked foods are very vulnerable if they are touched by fingers that have been contaminated by low numbers of salmonellas. Furthermore, any cooking process will have reduced the numbers of competitive organisms and thus the opportunity for subsequent multiplication of salmonellas is probably provided.

In the experiments to study the survival of *E. coli* and *S. anatum* on contaminated finger-tips there was a sharp fall in the percentage recovery of organisms in the 0–15 min. period after contamination. It was during this period, and usually within 5 min. that the inocula dried on the finger-tips. In replicate tests on the finger-tips of both authors the percentage recoveries of *E. coli* for various inocula were similar with respect to the range and median values obtained (Table 1). Similar tests, on the finger-tips of nineteen other persons gave different results; the percentage recovery rates and median values were significantly greater. Nevertheless, the numbers of organisms had fallen sharply 1 hr. after contamination. For *S. anatum* there was good agreement in the percentage isolation from finger-tips obtained in nine replicate tests on one of the authors and single tests on eleven other persons (Table 2). The results in Table 3 show that *S. anatum* could be isolated from the finger-tips for at least 3 hr. after contamination with an inoculum of about 500 organisms. It is also evident (Table 4) that there is no appreciable difference in the survival rates on skin of five of the six *Salmonella* serotypes studied.

Cooke *et al.* (1970) have reported that *E. coli* was present in 78 of 873 samples of food served to hospital patients. They suggested that many of the strains of *E. coli* had entered the hospital kitchen on raw meat and poultry and other foodstuffs, and had subsequently contaminated other raw foods and also cooked foods. We believe that salmonellas and *E. coli* can be and are transferred on many occasions by the hands from raw to raw and from raw to cooked or processed foods. Our results (Table 5) show that hand-washing with soap and water, followed by drying with paper towels, reduces the risk of transient skin carriage of salmonellas unless the initial contamination is very heavy. Much has been done in the past to encourage food handlers to wash their hands after visiting the w.c. A similar effort to encourage all food handlers to wash their hands after touching raw foods of animal origin is equally essential.

The results may also have some bearing on the mode of spread of *Salmonella* infection in hospitals. Short explosive outbreaks of foodborne *Salmonella* infection occasionally afflict hospitals, but more often the outbreaks run a protracted course over a variable period of time and therefore are less likely to have involved the continuous contamination of food and subsequent growth in it (Taylor, 1963; Williams, Blowers, Garrod & Shooter, 1966). However, even if multiplication in foods is not necessary, the salmonellas probably enter their new host by the mouth. The routes by which salmonellas travel from one patient to another have not been extensively studied, but these routes probably include the transient contamination of the hands of staff or patients (Mackerras and Mackerras, 1949; Watt *et al.* 1958),

and environmental contamination, e.g. fomites, dust and surfaces, which can act as secondary reservoirs (Rubbo, 1948; Parker, 1954; Bate & James, 1958; Rowe, Giles & Brown, 1969).

The ability of salmonellas to survive under various environmental conditions will affect their spread. Our results show that salmonellas can survive on the finger-tips for several hours and that during this time the hands can transmit infection.

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A biochemical subdivision of one phage type of *Salmonella typhimurium*

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SUMMARY

A total of 1537 strains of *Salmonella typhimurium* belonging to seven prevalent phage types were examined on solid media for their ability to ferment rhamnose, xylose and inositol, for colicine production and for nutritional requirements. Most of the strains in each phage type were almost completely homogeneous, especially in their sugar fermentation reactions. However, strains of phage type 1a/2 were not homogeneous, but could be assigned to one of four subgroups on the basis of ability to ferment inositol, inhibition of growth by *meso*-tartrate and auxotrophy for nicotinic acid. The subdivision proved to have epidemiological value. The inhibition of growth by *meso*-tartrate was observed on a defined medium containing citrate as the energy source. Inhibition did not occur if glucose, casein hydrolysate or aspartic acid were added to the medium.

INTRODUCTION

Salmonella typhimurium is the commonest salmonella in the United Kingdom, causing some 50,000 human infections annually and attacking large numbers of farm livestock (Anderson, 1965). Investigation of the epidemiology of this organism has been made possible only by the development of methods of identifying different strains within the serotype. The most widely used of these methods have been the phage typing schemes devised by the late Miss Bessie Callow (Felix & Callow, 1943; 1951, Callow, 1959). The first of these schemes (scheme 1) entails the use of 10 typing phages to distinguish 12 types and subtypes of *S. typhimurium*. It was soon apparent that a greater number of epidemiologically valid types existed, and in 1959 Miss Callow published a new typing scheme (scheme 2) distinguishing at that time some 80 types. For some years both schemes have been used in parallel in the Enteric Reference Laboratory, Colindale, and the number of recognized phage types has further increased (Anderson, 1965). In this paper we will refer to phage types of *S. typhimurium* by their designations in both schemes; for example, type 1a/2 belongs to the type designated 1a in scheme 1

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and 2 in scheme 2. A phage-typing scheme devised independently by Lilleengen (1948), depending on 12 anti-O phages to define 24 phage types, has been used in Scandinavia (Lundbeck, Plazikowski & Silverstolpe, 1955; Kallings & Laurell, 1957).

Strains of *S. typhimurium* differ in characters other than their reactions to typing phages. Kristensen, Bojlén & Faarup (1937) divided 361 strains into 18 types by tests of ability to ferment xylose, rhamnose, inositol and organic acids, and of ability to grow on defined media with citrate and tartrate isomers as sole carbon sources. This scheme was later extended to define 21 'fermentation types' which appeared stable and epidemiologically valid (Harhoff, 1948). Kallings & Laurell (1957) examined 393 strains from 120 outbreaks by both fermentation reactions and Lilleengen's typing phages. Some phage types correlated well with some fermentation types, but it was possible to subdivide 9 of 11 well-represented fermentation types by phage reactions, and 5 of the 14 well-represented phage types by fermentation pattern; this enabled strain identification to be carried beyond the point reached by either scheme alone.

In an investigation of strains of *S. typhimurium* of known phage type, provided by Dr E. S. Anderson (Enteric Reference Laboratory, Central Public Health Laboratory, Colindale) it was found (Stocker & Edgar, cited by Morgenroth & Duguid, 1968) that a minority of strains failed to grow on a defined medium with ammonium salts as sole nitrogen source, and that most of the nutritionally exacting strains grew well if provided with nicotinic acid or nicotinamide. Nearly all the strains requiring this growth factor were of Callow's phage type 1a/2. We describe here a survey of some variable biochemical characters in strains of *S. typhimurium* belonging to some of the commoner phage types, and the use of some of these characters, including nutritional character and the newly discovered property of sensitivity to *meso*-tartrate, for the subdivision of type 1a/2 into four biochemical subtypes – a subdivision which may have epidemiological value.

MATERIALS AND METHODS

Strains of Salmonella typhimurium

Dr E. S. Anderson kindly sent us 1537 strains of *S. typhimurium* selected from those submitted to the Enteric Reference Laboratory for phage typing in 1961 and 1962. These strains had been isolated from a variety of sources including cases of human and animal infection, drain and sewer swabs, and human and animal foods. They were selected from the seven more prevalent phage types and were as far as possible epidemiologically unrelated.

The strains were inoculated in a standard pattern on nutrient agar master plates, from which inocula were transferred by a multi-point replicator to solid media for the determination of sugar reactions, colicine production and nutritional character. Twenty-five test and two control strains were accommodated on each plate.

Media

Nutrient broth was prepared from a tryptic digest of beef, and was solidified with 15 g. Davis New Zealand agar per litre for plate culture, and 3·5 g./l. for soft agar.

The defined medium contained K_2HPO_4 10·5 g., KH_2PO_4 4·5 g., $MgSO_4$ 0·05 g., $(NH_4)_2SO_4$ 1 g., sodium citrate 0·47 g., glucose 2 g., Davis agar 15 g., and distilled water to 1 l. This medium was supplemented, when necessary, with nicotinic acid 1 μg./ml. In one series of tests the glucose was replaced by 5 g./l. of glycerol, to determine the ability of strains to use this substance as a source of carbon and energy. When testing strains for ability to grow in the presence of *meso*-tartrate, glucose was omitted from the defined medium, the concentration of sodium citrate was increased to 5 g./l. and 5 g./l. of sodium *meso*-tartrate were added.

Solid medium for the determination of sugar fermentation reactions contained Evans bacteriological peptone 20 g., NaCl 5 g. and Davis agar 15 g. in 925 ml. water. The initial pH was 7·2. Immediately before use this peptone agar was melted and the following solutions were added: 10% sodium deoxycholate, 25 ml.; 1% neutral red, 2·5 ml.; 20% sugar, 50 ml. On this medium strains which ferment the sugar produce red colonies, while those which do not ferment the sugar produce opaque cream colonies. All plates were incubated at 37° C. and scored after 24 and 48 hr. incubation.

Tests for the production of colicine

Replicates of the master plate cultures were made on tryptic digest agar and incubated overnight at 37° C. The bacteria were killed with chloroform vapour and the plate covered with a layer of soft agar seeded with about 10^8 bacteria of an indicator strain. The indicator strains used, sensitive to all the common colicines (Ozeki, Stocker & Smith, 1962) were CL 142 (i.e. *E. coli* K12-row of Fredericq) and CL 104 (a streptomycin-resistant derivative of *E. coli* strain ϕ).

RESULTS

Sugar fermentation tests

All 1537 strains of *S. typhimurium* were inoculated from the master plates to rhamnose, xylose and inositol fermentation plates.

Rhamnose fermentation

On rhamnose medium the strains were clearly differentiated into those able to ferment this sugar vigorously and those unable to ferment it at all. The rhamnose-fermenting strains gave deep red colonies, each surrounded by an opaque red zone about 0·5 cm. wide in which acid diffusing into the medium had changed the colour of the indicator and caused precipitation of deoxycholic acid. These strains have been termed 'Rha-A'. Those unable to ferment rhamnose (Rha$^-$) showed no redness in the colony and no change in the surrounding medium. Of the 1537 strains tested, 1004 were Rha-A and 533 Rha$^-$.

Xylose fermentation

On xylose medium the results were more complex. One hundred and seventy strains gave a reaction like that described above as 'Rha-A', i.e. deep red colonies with a surrounding colour change and precipitate in the medium; these have been termed 'Xyl-A'. Three strains were unable to ferment xylose (Xyl$^-$). The colonies of the remaining 1364 strains, although red, were not as deeply red as those of the Xyl-A strains, and there was no change in the appearance of the medium surrounding them. This appearance was attributed to a fermentation of xylose less vigorous than that effected by the Xyl-A strains, and was recorded as 'Xyl-a', to indicate the weaker acid reaction.

Inositol fermentation

None of the strains tested fermented inositol vigorously, as judged by appearances on the inositol indicator agar plates; to obtain consistent differentiation between fermenting and non-fermenting strains it was necessary to force the prongs of the replicator into the medium until they struck the bottom of the plate, so that growth occurred on the surface of the agar, in the depth of the stab and as a film in the interface between the agar and the bottom of the dish. Under these

Table 1. *Fermentation of Rhamnose, Xylose and Inositol by* 1537 *strains of* Salmonella typhimurium

Sugar fermentation pattern			No. of
Rha	Xyl	Ino	strains
A	A	a	170
A	a	a	193
A	a	—	638
A	—	—	3
—	a	—	533
Total			1537

A, Strong acid production on sugar indicator agar plates. a, Weak acid production. —, No acid production.

conditions 363 strains gave red colonies corresponding to the 'weak' type of fermentation. The colour was most apparent in the film of growth in the interface between the agar and the bottom of the plate, but extended into the surface growth. Scoring was unequivocal when the colonies were viewed through the bottom of the dish. These strains were recorded as 'Ino-a'. It is likely that the stronger acid reaction in the depth of the medium results from the partially anaerobic conditions in that part of the colony. The remaining 1174 strains showed no evidence of acid production, and were recorded as 'Ino$^-$'.

Table 1 records the fermentation of these sugars. Five fermentation patterns suffice to describe all the 1537 strains tested.

Nutritional characters

The nutritional requirements of these strains were examined by replicating from the master plates to three sets of plates of the defined medium. In one set the carbon source was 0·2% (w/v) glucose with 0·047% citrate. In another, the glucose was replaced by 0·5% (w/v) glycerol. In the third, the glucose was omitted and the citrate concentration increased to 0·5%. A total of 1334 strains were able to grow well on all these media. Six strains grew poorly or not at all on the plates containing glycerol, and one was unable to utilize citrate. Of the remaining 196 nutritionally exacting strains 174 needed nicotinic acid. These will be termed Nic$^-$. A further 10 grew well when the media were supplemented with a pool which included thiamine, riboflavin, biotin, pantothenate and pyridoxin, and 12 when supplied with 0·1% casein hydrolysate. We did not examine these last 22 strains for their ability to grow on media supplemented with individual growth factors or amino acids.

Colicinogeny

The incidence of colicinogeny in this series of strains was low, 1426 strains producing no colicine active on *E. coli* K12 or on *E. coli* ϕ. Of the colicinogenic strains, 105 produced a colicine I, 3 colicine E 1, 2 colicine V and 1 strain an unidentified colicine.

The distribution of the characters we have been considering among the very small number of phage types represented in this series revealed that most of the phage types were remarkably homogeneous, especially in their sugar fermentation reactions. Thus, 152 of 154 strains recorded as belonging to phage type 2/12a had the fermentation pattern Rha-A Xyl-A Ino-a; 532 of 541 strains of type 2c/14 were unable to ferment rhamnose or inositol, and all gave the Xyl-a reaction; 206 of 209 strains belonging to type 1 var 5/U9 gave the pattern Rha-A Xyl-a Ino$^-$. The incidence of colicinogeny was less than 18% in any group. Except within phage type 1a/2 the incidence of auxotrophy was so low as to make subdivision by this character unrewarding. However, it was also apparent that strains belonging to phage type 1a/2 were not homogeneous in respect of the characters tested.

Biochemical differentiation within phage type 1a/2

There were 315 strains belonging to phage type 1a/2 in this series. All except one of these strains fermented rhamnose (Rha-A), and all gave the Xyl-a reaction. But only 38 strains were found to ferment inositol, and 163 were Nic$^-$.

During an investigation of the ability of strains of *S. typhimurium* to utilize organic acids as sources of carbon it was noticed that some strains which grew well on a simple defined medium did not grow, or grew very poorly, when *meso*-tartrate was present in the medium. The degree of inhibition was greatly influenced by the composition of the basal medium, and the most consistent results were obtained by using the *meso*-tartrate medium described in the methods section. On this medium strains sensitive to *meso*-tartrate showed no growth after 48 hr.

incubation. Of 290 strains of phage type 1 a/2 tested, 44 were inhibited by *meso*-tartrate.

When the characters we have been discussing were correlated four major groups emerged (Table 2). Thirty-six strains fermented inositol, were resistant to *meso*-tartrate and were Nic$^+$ (group 1). Sixty-four strains were Ino$^-$, *meso*-tartrate-resistant and Nic$^+$ (group 2). Forty-two strains were Ino$^-$, sensitive to *meso*-tartrate and Nic$^+$ (group 3). One hundred and forty-four strains were Ino$^-$, *meso*-tartrate-resistant and Nic$^-$ (group 4).

Table 2. *Biochemical subdivision of* 290 *strains of* Salmonella typhimurium *phage type* 1 *a*/2

Group	Rha	Xyl	Ino	*Meso*-tartrate	Nic	No. of strains
1	A	a	a	R	+	36
2	A	a	—	R	+	64*
3	A	a	—	S	+	42†
4	A	a	—	R	—	144
Other	—	a	—	R	—	1
	A	a	—	?	—	1‡
	A	a	a	R	—	1
	A	a	a	S	+	1

Sugar reactions: A, Strong acid production on sugar indicator agar plates; a, weak acid production; —, no acid production.

Meso-tartrate: R, resistant; S, sensitive to inhibition by *meso*-tartrate, tested by ability to grow on a defined medium with citrate as the energy source and containing 5 g./l. sodium *meso*-tartrate.

Nic: +, able to grow on a medium devoid of nicotinic acid; —, unable to grow in the absence of nicotinic acid.

* One strain auxotrophic for thiamine.

† Two strains unable to utilize glycerol.

‡ This strain cannot utilize citrate.

All except four of the 290 strains fell into one or other of these groups. One of the four, which in all other respects belonged to group 4, was unable to ferment rhamnose. One was unable to use citrate as a carbon source, and thus could not be scored for tartrate sensitivity, but as it was Nic$^-$ it probably belongs to group 4. The other two anomalous strains were both able to ferment inositol, but one was Nic$^-$ and the other was inhibited by *meso*-tartrate. In addition, one strain in group 2 was unable to grow in the absence of thiamine, and two strains in group 3 were unable to utilize glycerol as sole carbon source. These results are shown in Table 2.

The suggestion that these groups represent epidemiologically valid subtypes of phage type 1 a/2 received support from the examination of 74 additional strains of this type. All were isolated within a period of a few weeks from what at first appeared to be a single widespread outbreak involving adjacent areas of the counties of Lancashire and Yorkshire. Biochemical tests showed that 37 of these strains belonged to group 4, and all these had been isolated in Lancashire; 36

strains belonged to group 2, and had been isolated in Yorkshire; one strain, also from Yorkshire, belonged to group 3. Epidemiological assessment in the light of these findings suggested that we were observing two simultaneous outbreaks – a group 4 outbreak centred on the city of Manchester and a group 2 outbreak centred on Leeds, and that the one strain belonging to group 3 was from a sporadic case unconnected with either outbreak. We are grateful to Dr E. S. Anderson, of the Enteric Reference Laboratory, for giving us the opportunity of examining these strains during the development of the outbreak.

Inhibition of Salmonella typhimurium *by* meso-*tartrate*

The observation that some strains of *S. typhimurium* which are able to grow well on a simple defined medium are unable to grow when *meso*-tartrate is added demanded further investigation. No strains were found to use *meso*-tartrate as a sole source of carbon, and the inhibition of sensitive strains was to some extent dependent on the other carbon sources present. On defined media with citrate, glycerol, maltose or lactate as sole carbon sources there was consistent and complete inhibition, but inhibition was neither complete nor consistent when the medium contained glucose, glucose and citrate, or galactose.

Growth was not inhibited by *meso*-tartrate when as little as 0·01 % of casein hydrolysate was added to the medium, but it proved remarkably difficult to demonstrate that this effect was due to the action of any particular amino acid or combination of amino acids. The L isomers of alanine, glycine, serine, leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophane, lysine, arginine, methionine and cysteine were added to the medium alone and in various combinations, in concentrations up to 0·1 %, but none caused any reversal of inhibition by *meso*-tartrate. When glutamic acid, proline or histidine were added to the medium sensitive strains were still inhibited after 18 hr. incubation, but showed some growth, not as vigorous as that of the uninhibited strains, after 48 hr. Aspartic acid, alone among the amino acids, caused a significant, although not complete, reversal of the effect of *meso*-tartrate within 18 hr., and complete reversal after 48 hr. incubation.

The reversal of tartrate inhibition by these amino acids may be related to the readiness with which bacteria can use them as sources of carbon and energy. Proline, glutamic acid and aspartic acid can all replace glucose in the minimal medium, although they do not induce as vigorous growth as does glucose. But proline is superior to aspartic acid in this respect, although much less effective in reversing inhibition by *meso*-tartrate, while histidine is a very poor carbon and energy source.

DISCUSSION

A common and widespread bacterial species can be expected to evolve into subspecies varying in any of a number of different characters, such as ability to ferment a sugar or to be attacked by a phage or to produce different antigens. Different epidemiologically valid typing schemes can be devised using any variable

characters of a bacterial species to define groups of strains (types) which are alike in the characters studied.

Duguid, Anderson & Campbell (1966) drew attention to a correlation between the characters of fimbriation and rhamnose fermentation in *S. typhimurium* which led to the description of two groups of Rha$^-$ strains of this organism. In the first group, designated FIRN and of frequent occurrence, the Rha$^-$ character is accompanied by failure to ferment inositol and inability to produce fimbriae. The second, or non-FIRN, group contains all other Rha$^-$ strains; these are relatively uncommon. Morgenroth & Duguid (1968) have shown that the sites of the Rha$^-$ mutations differ in the FIRN and non-FIRN groups, but that within each group the mutation appears to be located at the same site in every strain.

In another study Stocker & Edgar (1959) showed that 15 wild Nic$^-$ strains of *S. typhimurium* of phage type 1a/2 all appeared to be mutant at the same site, different from but linked to the site of mutation of a Nic$^-$ line of *S. typhimurium* LT 2 derived in the laboratory.

It is likely that all strains in any one of these groups are descendants of a single bacterium (that is, each group is a clone), and this supposition is strengthened by the fact that the strains have other characters in common. However, mutations, even at identical loci, can occur in different lines at different times, and a single genetic change might result in alteration in several apparently unrelated characters; for example, a mutation affecting carbohydrate metabolism may alter the antigenic structure of a bacterium, and consequently affect its sensitivity to phages. Such a change may be reflected in differently based typing schemes, suggesting a spuriously close relationship between strains of diverse origins.

The reactions of bacterial strains to typing phages may be determined, at least in part, by the carriage of temperate phages, or of bacterial plasmids such as R factors and colicinogenic factors, or of the transfer factors often associated with such plasmids (Anderson & Lewis, 1965; Anderson, 1966). Acquisition or loss of these agents provides another means by which originally similar strains may come to differ, or different strains come to resemble one another.

The use of two or more typing methods may often allow a greater precision in strain differentiation than can be achieved by using one method alone, at the expense of an increase in complexity which may become self-defeating. Typing schemes must be practical. We suggest, however, that a limited use of biochemical characters may be of value in subdividing common phage types of *S. typhimurium*, and we describe one such subdivision for one phage type of this serotype.

The use of the multipoint replicator greatly reduced the work involved, a consideration of importance when many strains have to be typed in an epidemic, and the testing of sugar fermentation reactions on a solid indicator medium permitted the differentiation of 'strong' and 'weak' fermentation of xylose, a distinction which cannot be made when using peptone-water sugar media.

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A comparison of the iodine and fluorescent antibody methods for staining trachoma inclusions in the conjunctiva*

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SUMMARY

In terms of the rate of positive diagnoses the indirect fluorescent antibody (FA) test was rather more effective than iodine for demonstrating trachoma (TRIC) inclusions in conjunctival scrapings, but the degree of advantage was not statistically significant. In duplicate scrapings stained at random by one or the other method, FA staining yielded the higher inclusion count significantly more often than did iodine. Some inclusions that failed to stain with FA were found on subsequent staining with Giemsa. A method is described for improving the post-FA Giemsa staining of conjunctival smears stored at subzero temperatures. Given adequate facilities, the FA stain is preferable to iodine for demonstrating TRIC inclusions in the conjunctiva; but the iodine method, properly used, holds advantages for field use.

INTRODUCTION

The problem of which is the 'best' method for detecting trachoma/inclusion conjunctivitis (TRIC) agent in the conjunctiva has long been a matter of controversy; and perhaps the reason why there is no clear-cut answer lies in the diversity of ends to which the various means are directed. Whereas one worker may be interested in the rapid screening of large populations, another may be concerned only with an occasional diagnostic test, or with careful observation of an experimental infection in a few human volunteers or monkeys.

The *Chlamydia*, including TRIC agents, are demonstrable by direct microscopy of infected cells, in which they form cytoplasmic inclusion bodies of characteristic morphology and staining reactions; and by isolation in a suitable host. Halberstaedter & von Prowazek (1907) first observed trachoma inclusion bodies in conjunctival scrapings stained with Giemsa, and although other methods have been described, this stain probably remains the most popular. It reveals the varying morphology and staining characteristics of the inclusion at all stages of development, its relation to the cytoplasm and nucleus of the host cell and the presence of free elementary bodies. The disadvantages are that conjunctival smears must be scanned with the higher-power objectives – a slow and laborious process; that inclusions are often missed because they are hidden in densely

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stained clumps of cells; and that it may be difficult or impossible to decide whether a basophilic granule in the cytoplasm is an early inclusion or ingested debris.

During the later stages of development the inclusions of type A chlamydiae contain a carbohydrate matrix that stains brown with iodine and is closely similar to, if not identical with, glycogen (Rice, 1936). It is probably true that the iodine method has been exploited more extensively by the M.R.C. Trachoma Unit than by any other group of workers. In 1958 Gilkes, Smith & Sowa described the use of dry iodine-stained smears covered with a thin layer of immersion oil. This technique gave better definition than the wet preparations used hitherto, and proved particularly useful for detecting faintly stained inclusions; it was used extensively in the study of experimental infections in man (Collier & Sowa, 1958; Collier, Duke-Elder & Jones, 1958, 1960; Jones & Collier, 1962) and in baboons (Jones, Collier & Smith, 1959; Collier, 1961, 1962; Collier & Blyth, 1966*a*, *b*; Collier, Blyth, Larin & Treharne, 1967).

One of us (J.S.) devised a modification in which the use of weak ammonia improved the contrast between iodine-stained inclusions and the background, and minimized staining of red cells and leucocytes (Sowa, Sowa, Collier & Blyth, 1965). This method was used in the investigation described in the present paper. The iodine method has several advantages: staining takes but a few minutes, and can easily be done in the field; it reveals inclusions buried within clumps of epithelial cells, so that thick smears containing large numbers of cells can be examined with ease; the characteristic colour and granular morphology of the inclusions leaves but little room for error in interpretation; and with experience a conjunctival scraping can be scanned completely in 10 min. with a $\times 10$ or $\times 20$ objective, with virtually no risk of missing even a single stained inclusion. The only disadvantage is that at some stages in their development inclusions do not stain with iodine; but it must be rare for all the inclusions in a given scraping to fall into this category.

The indirect fluorescent antibody method was used 16 years ago to study the growth cycle of a chlamydia in cell cultures (Buckley, Whitney & Rapp, 1955) but it was not until 1961 that Vozza & Balducci attempted to stain trachoma inclusions in conjunctival scrapings by this method, with inconclusive results. In the following year Siboulet, Galistin & Huriez (1962) published a short note on the use of this technique for diagnosing inclusion blennorrhoea. The application of immunofluorescence to the diagnosis of TRIC infections was first placed on a firm footing by Nichols and his colleagues (Nichols & McComb, 1962; Nichols, McComb, Haddad & Murray, 1963); these workers exploited the technique for large-scale studies of trachoma in Saudi Arabia (Nichols, Bobb, Haddad & McComb, 1967). Immunofluorescence was used by investigators at the University of California Medical Center to diagnose TRIC infections in patients and in artificially infected volunteers (Hanna *et al.* 1965; Hanna, 1968), and for assessing the results of treatment (Dawson, Hanna & Jawetz, 1967). The California workers also claimed that immunofluorescence revealed inclusions in a surprisingly high proportion of American Indian schoolchildren with no signs of trachoma, or with signs of inactive disease only (Jawetz *et al.* 1967).

Although several workers compared immunofluorescence with the Giemsa method for detecting conjunctival inclusions, we have found only one report of its comparison with the iodine technique (Hanna *et al* 1965); this is referred to in the Discussion. The researches we describe were designed primarily to assess the relative efficacy of the fluorescent antibody and iodine stains in terms of their suitability both for limited studies and for large-scale screening procedures; they formed part of a series of investigations of trachoma in young Gambian children.

MATERIALS AND METHODS

General plan

The investigations were undertaken in the large Gambian village of Salekini (Sowa, Sowa, Collier & Blyth, 1969). During 1968, preliminary tests were made on 36 children with active trachoma in order to perfect techniques (study no. 1). In January 1969 a group of 99 children aged 3 years and under was assembled. Of these, 37 had normal (N) eyes, 34 had active trachoma (Tr I or II), 10 had physical signs suggestive of early trachoma (Tr D), and 18 had minor conjunctival abnormalities not suggestive of trachoma (Ab). The children were tested 5 times between January and October 1969; a few were absent from one or more examinations. On each occasion their eyes were carefully examined with a slit-lamp, and conjunctival scrapings were taken for microscopy. In addition, samples of conjunctival secretions and blood were tested for antibody; these findings will be reported in a further communication.

Conjunctival scrapings

The conjunctivae were anaesthetized by instilling 0·5% 'Amethocaine' and 0·001% adrenalin hydrochloride. The tarsal area of the everted upper lid was then lightly scraped with a small spatula made by flattening one end of a length of $\frac{1}{16}$ in. (1·5 mm.) aluminium welding rod; scrapings from each eye were spread over separate areas on a microscope slide. When comparing 'thick' with 'thin' smears, a light scraping was taken from the medial half of the tarsal conjunctiva for the 'thin' specimen, and a rather more vigorous scraping from the lateral half for the 'thick' smear. Thin smears were made by spreading a small quantity of material over an area approximately 10×2 mm. For thick smears, about 10 times more material was spread over a circular area 10–15 mm. in diameter; this is our routine practice for the iodine method (Sowa *et al.* 1965). Duplicate thin smears for comparing iodine with fluorescent antibody staining were made by dividing a single epithelial scraping from the mid-tarsal area between two slides which were stained at random by one or the other method.

Iodine stain

Air-dried smears were flooded with absolute ethyl alcohol, drained and allowed to dry. They were then treated for 2 min. with 88 vol. ammonia freshly diluted to 1/200. The ammonia was then rinsed off with a small quantity of ethyl alcohol; this step requires caution since the ammonia tends to loosen the material on the

slide. It was found best to stain slides individually with ammoniated iodine (see below) shortly before examination.

After applying iodine for at least 2 min. the excess stain was poured off and the slide was immediately blotted dry on filter paper. After delineating the smear with a wax pencil, the entire area was systematically scanned with a $\times 10$ objective (final magnification $\times 120$) and the total number of inclusions was counted. In the 'dry' method originally described by Gilkes *et al.* (1958) the stained smears were covered with a thin layer of immersion oil. The use of oil to improve contrast depends on the optical properties of the microscope used; it was not necessary in this investigation.

Ammoniated iodine

The following stock solutions were prepared: A, saturated iodine solution in 20% (w/v) potassium iodide; B, potassium iodide 20% (w/v); C, 88 vol. ammonia, diluted 1/20 in distilled water.

To prepare the stain, 0·5 ml. of A was added to 4·5 ml. of B and mixed well. After rapidly adding 5 ml. of C the mixture was shaken vigorously. Any precipitate was removed by sedimentation or filtration. (Note that dried ammonium iodide is fulminant.) This solution is best kept in darkness, and should not be stored more than 3 days.

Giemsa stain

This stain was used to verify the morphology of inclusions previously stained with FA. To 50 ml. of phosphate buffer pH 6·8 were added 1 ml. of Giemsa R 66 and 0·5 ml. of May–Grünwald stain (G. Gurr and Co., London). The slides were immersed in this solution for 1 hr., rinsed in buffer and dried.

Fluorescent antibody (FA) methods

Diluent. All sera and slide antigens were diluted in phosphate-buffered saline (PBS) pH 7·2 (Fothergill, 1964).

Fixation and storage of scrapings. In surveys 1 and 2, slides were fixed for 10 min. in acetone at room temperature and then stored at $-20°$ C. or below until examination. In surveys 3–6 they were stored at $-20°$ C. or below and fixed in acetone just before staining. There was no evidence that this variation affected the results.

Antiserum to TRIC agent grown in yolk sac was prepared by injecting a rabbit intravenously with a partly purified suspension of live TRIC/2/GB/MRC-4/ON (formerly LB4: Jones, 1961; Jones & Collier, 1962) in its 8th chick embryo passage. This is a 'slow-killing' strain (Reeve & Taverne, 1963); the abbreviation is MRC-4*s*. The suspension contained $10^{6\cdot0}$ 50% egg lethal doses and $10^{10\cdot0}$ total elementary bodies in 1 ml.; it was stored in 1 ml. volumes in liquid nitrogen. The rabbit received four 1 ml. doses at weekly intervals; after 10 weeks rest it was given a booster dose. Serum obtained by heart puncture 1 week after the final dose was stored in 0·1 ml. volumes at $-60°$ C. Its complement-fixing titre against *Chlamydia* group antigen prepared from infected HeLa cells (Collier & Blyth, 1966*a*) was 1/1280.

Antisera to TRIC agent grown in cell culture. Strain MRC-4*s* was propagated in BHK-21 cells by centrifuging the agent into monolayers (Taverne & Blyth, 1971), incubating at 35° C. for 48 hr., disrupting the monolayer with ultrasonic vibrations, and using the resulting suspension to infect the next lot of cells. After three preliminary passages to eliminate any yolk-sac antigen, a sonic-treated suspension of heavily infected cells was made and stored at −60° C. in 1 ml. volumes each containing $10^{8\cdot1}$ elementary bodies and $10^{4\cdot3}$ inclusion-forming units. Two rabbits (nos. 366 and 367) each received four 1 ml. doses at weekly intervals and were bled out 1 week after the final dose. The complement fixation titres of the sera against group antigen prepared in yolk sac were 1/160 and 1/320 respectively.

Anti-rabbit serum prepared in goats and conjugated with fluorescein isothiocyanate was obtained from Sevac Laboratories.

Staining of conjunctival scrapings. Preliminary chess-board titrations with conjunctival scrapings containing many inclusions indicated that the optimum dilutions of anti-TRIC and anti-rabbit sera were 1/20 and 1/80 respectively, and these dilutions were used as a routine. Smears were covered with diluted antiserum and incubated at 37° C. for 30 min. in a moisture-saturated atmosphere. After rinsing off excess serum, the slides were rinsed in 50 ml. of PBS and dried in a current of cold air. After repeating the procedure with anti-rabbit conjugate the smears were mounted under coverslips in glycerol-PBS, 9:1.

Staining of yolk-sac antigens. Slide antigens were prepared by placing a series of small loopfuls of a 5% yolk sac suspension on a slide, drying in air, and fixing in acetone for 10 min. at room temperature; these slides were stored at −30° C. Sera were titrated by covering each spot with an appropriate dilution of serum and incubating the slides for 30 min. at 37° C. in a humidity box. After washing off the sera with PBS delivered from a wash-bottle, and two further 5 min. rinses in PBS, the drops were dried in an air stream, and then covered with a 1/40 dilution of goat anti-rabbit serum conjugated with fluorescein isothiocyanate; this dilution was chosen by chess-board titration of the conjugate with serum 367 and the homologous yolk sac antigen. The incubation and washing procedures were repeated, except that 5 drops of 0·1% Evans blue were added to 100 ml. of PBS for the final rinse. (The use of this counterstain was described by Nichols & McComb (1964), but in a higher concentration.) The antigen spots were mounted under cover-slips in glycerol-PBS. Each slide included two control spots treated respectively with buffer only, and buffer followed by conjugate. When handling more than a few slides at once, we found it very convenient to mount them in plastic storage holders (Bie and Berntsen, Copenhagen, Denmark). The use of these frames enables 12 slides to be stained, incubated and rinsed together.

Ultraviolet microscopy was done with a Zeiss Photomicroscope equipped with an HBO 200 lamp. Slides were scanned under bright field illumination with exciter filter BG 12/4 and barrier filter 53; the objective was a ×40 apochromat. Unless there were more than 50 inclusions, the entire area of each conjunctival scraping was scanned. The brightness of stained yolk-sac antigens was assessed with a set of graded density filters (Collier, 1968). The titration end-point was taken as the first doubling dilution (reading from the lowest) to give a score of 1.

Verification of FA stained inclusions with Giemsa

In study no. 1 and in the 1st survey of study no. 2, all inclusions or probable inclusions stained with fluorescent antibody were photographed on 35 mm. Kodak Tri-X film after recording their position on the stage micrometer. The slide was then stained with Giemsa; to identify the inclusions previously photographed under u.v. light, the photographic negative was projected on a screen and compared directly with the corresponding Giemsa-stained field under the microscope.

Coding of slides

Throughout this investigation all conjunctival scrapings were identified by code number only; one microscopist examined the FA stained smears and another the iodine-stained preparations. Neither was aware of his colleague's findings, or of the results of the clinical examinations.

RESULTS

Tests on TRIC antiserum

Specificity for TRIC antigen. From each eye of two trachoma patients, a thick smear and two thin smears of conjunctival scrapings were made. The thick smear was stained with iodine. One of the thin smears was stained by the routine FA method; the other was stained in the same way, except that normal rabbit serum was substituted for the anti-MRC-4*s* serum. Inclusions were found only in the iodine-stained scrapings, and in those stained with the anti-MRC-4*s* serum (Table 1).

Table 1. *Conjunctival scrapings from two trachoma patients stained by the immunofluorescence (FA) method or by iodine*

	No. of inclusions in scrapings stained by		
	FA		
Patient and eye	Normal serum	Anti-TRIC serum	Iodine*
135 R	0	0	6
135 L	0	4	20
166 R	0	5	10
166 L	0	2	16

* Thick smears containing about 10 times more material than FA-stained smears.

Heterogeneity for TRIC serotypes. The anti-TRIC serum used for staining inclusions was prepared against strain MRC-4*s*, a type 2 TRIC agent. Since the rabbit was immunized by repeated doses, and the serum was not cross-absorbed, it should have been capable of staining inclusions resulting from infection with any TRIC serotype. Because this serum reacted strongly with yolk sac, it proved difficult to verify this supposition by testing against our set of antigens represent-

ing the various serotypes, all of which had been propagated in yolk sac. Accordingly, sera were prepared against MRC-4*s* grown in BHK-21 cells and titrated against the antigens indicated in Table 2. The titres of serum 367 were higher than those of 366 (which also had the lower titre of complement-fixing antibody); but it is apparent from Table 2 that both sera reacted adequately with all the major TRIC serotypes, and it seems reasonable to assume that the anti-MRC-4*s* serum actually used for staining conjunctival material possessed a similar degree of heterogeneity.

Table 2. *Fluorescent antibody titrations of anti-MRC-4s sera with various TRIC serotypes*

		Serum no.	
Serotype	Strain	366	367
1	SAU/HAR-13/OT	20*	160
1b	SAU/HAR-32/OT	20	160
2	SAU/HAR-36/OT	40	160
2	GB/MRC-4*s*/ON	80	320
D	GB/MRC-1/OT ('G 1')	20	160
E	USA-Cal/Cal-1/OT ('BOUR')	40	160
F	USA-Wash/UW-6/GCx	20	40
.	Normal yolk-sac control	< 10	< 10

* Reciprocal of end-point dilution.

Criteria for validity of FA staining of inclusions

No scraping was scored as positive by FA staining unless it contained morphologically characteristic inclusions that were contained within the intact cytoplasmic boundaries of epithelial cells, and that stained brightly with a yellow-green fluorescence (Plate 1 *a–d*). Almost all patients diagnosed as inclusion-positive by FA staining were also positive by the iodine or Giemsa methods. In study no. 1 for example, of 15 patients diagnosed as inclusion-positive by FA, 12 were also positive by both the other methods.

Relative efficacy of iodine and FA stains

Study no. 1. Comparison of the FA method with the iodine technique used by us as a routine is not straightforward; the former demands a thin smear, whereas a much larger sample of cells is examined in the thick smear used in the iodine method. It was thus desirable in the first instance to compare the results of staining duplicate thin smears by both methods with those of tests on thick smears stained by iodine alone. At this stage the numbers of inclusions were not counted, and results were recorded merely as positive or negative. Tables 3 and 4 give the findings in 36 children with active trachoma. Somewhat to our surprise the three methods (thick smear stained with iodine, thin smear stained with iodine and thin smear stained with FA) yielded similar numbers of positive tests (Table 3) although the individuals diagnosed as inclusion-positive by one or the other method did not correspond exactly. The proportion of inclusion-positive patients was about 15/36 (42%) with any one method. With a combination of any two

methods about 50% were positive; and with all three methods this figure rose to 21/36, or 58% (Table 4).

The percentages of positive and of false negative results were similar with all three methods, so that failure of correspondence in a proportion of findings was probably attributable to sample variation; in other words, use of thin smears only for comparing the diagnostic efficacy of the iodine and FA methods would not bias the results unduly against the iodine test.

Table 3. *Study no. 1: comparison of results of tests for inclusions in thick and thin smears stained with iodine, and thin smears stained with fluorescent antibody; numbers of inclusion-positive eyes*

	No. of eyes		
	Tk I positive	Tk I negative	Totals
Tn I positive	12	8	20
Tn I negative	10	42	52
Totals	22	50	72
FA positive	12	9	21
FA negative	10	41	51
Totals	22	50	72
	Tn I positive	Tn I negative	
FA positive	13	8	21
FA negative	7	44	51
Totals	20	52	72

Tk I = thick smear stained with iodine. Tn I = thin smear stained with iodine. FA = thin smear stained with fluorescent antibody.

Table 4. *Study no. 1: numbers of patients diagnosed as inclusion-positive by combinations of the three methods*

		Additional methods		
	Alone	FA	Tn I	FA and Tn I
Tk I	15	18	18	21
Tn I	14	19	.	.
FA	15	.	.	.

Abbreviations as in Table 3.

Study no. 2. As described under Materials and Methods, duplicate thin smears were made on 5 occasions from the 99 children participating (Table 5), and stained at random either with iodine or FA. Table 6 classifies the results according to clinical diagnosis, and Table 7 summarizes the findings in all patients who were inclusion-positive by either or both methods in each of the five surveys. In 448 examinations of individuals, at which Tr I or Tr II was diagnosed 189 times

Table 5. *Study no. 2: dates of surveys undertaken in* 1969

Survey	From	To
1	6 January	21 January
2	2 March	8 March
3	17 April	5 May
4	7 June	14 June
5	25 September	10 October

Table 6. *Study no. 2: comparative efficacy of iodine and FA staining for detecting trachoma inclusions; combined results for surveys* 1–5 *analysed by clinical diagnosis*

Clinical diagnosis*	Total no. of examinations	Tn I −, FA −	Tn I +, FA +	Tn I −, FA +	Tn I +, FA −	% inclusion-positive (either method)
N	111	111	0	0	0	0·0
Ab	88	86	2	0	0	2·3
Tr D	44	42	2	0	0	4·5
Tr I	62	53	7	2	0	14.5
Tr II	127	103	17	5	4	20·5
Tr III	16	13	1	2	0	18·8
Totals	448	408	29	9	4	9·4

* See text for meaning of these abbreviations; other abbreviations as in Table 3.

Table 7. *Study no. 2: comparative efficacy of iodine and FA staining for detecting conjunctival inclusions*

(Surveys 1–5 analysed by numbers of positive subjects and numbers of positive eyes.)

Survey no.	Positive (+) or Negative (−)	No. of patients		No. of eyes	
		Tn I	FA	Tn I	FA
1	+	7	9	11	11
	−	3	1	9	9
2	+	1	1	1	1
	−	1	1	3	3
3	+	4	5	5	9
	−	2	1	7	3
4	+	8	8	13	15
	−	0	0	3	1
5	+	13	15	23	28
	−	3	1	9	4
Totals	+	33	38	53	64
	−	9	4	31	20
		42	42	84	84
χ^2 (with Yates's correction)		1·456		2·815	
P		> 0·10		< 0·10, > 0·05	

Abbreviations as in Table 3.

(Table 6), the result was positive both by iodine and FA on 29 occasions; on 9 occasions the result was FA-positive but iodine-negative and on 4 it was iodine-positive but FA-negative. In 84 tests on individual eyes there were 31 false negative results with iodine, and 20 with FA. Although the proportion of positive

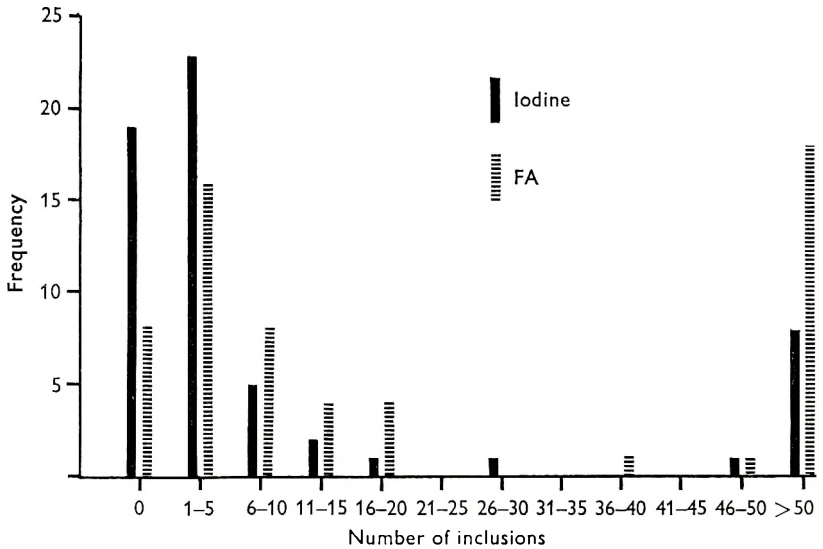
Table 8. *Study no. 2: comparison of numbers of inclusions in duplicate thin smears stained by iodine or by FA*

Survey no.	Patient no.	Nos. of inclusions in Right eye Tn I	Right eye FA	Left eye Tn I	Left eye FA
3	3	0	1	0	0
	7	0	0	1	0
	20	4	8	0	1
	27	5	5	1	> 50
	54	0	6	0	1
	73	0	5	2	> 50
4	2	3	9	1	2
	7	27	6	0	1
	10	3	12	2	4
	20	19	20	5	52
	27	7	46	14	10
	31	3	18	0	0
	61	0	2	1	14
	73	> 50	> 50	6	40
5	1	0	6	0	12
	3	0	2	0	1
	7	0	16	0	1
	10	0	2	3	2
	18	3	0	11	2
	22	> 50	> 50	> 50	> 50
	31	1	2	4	> 50
	32	> 50	> 50	> 50	> 50
	41	> 50	> 50	> 50	> 50
	46	0	0	1	0
	61	1	9	0	14
	73	3	> 50	6	> 50
	80	3	6	5	17
	88	46	> 50	1	0
	89	3	> 50	8	> 50
	93	10	> 50	> 50	> 50

Abbreviations as in Table 3.

results with FA was higher, it was not significantly so (Table 7). In surveys 3, 4 and 5 the number of inclusions in each smear was counted. When it exceeded 50, the result was usually recorded as '> 50' since the accurate counting of large numbers of inclusions was very time-consuming. Table 8 gives the actual inclusion counts made in surveys 3, 4 and 5 and Text-fig. 1 their distribution in histogram form. In this series of 60 scrapings taken in duplicate, the inclusion count in the FA-stained scrapings was recorded as the same as in the iodine-stained sample in

13 instances, including 4 in which both samples were negative. The count was greater in the FA-stained sample on 39 occasions, and less on 8 occasions; if there were no differences between the two techniques these numbers would be equal or nearly so. The observed departure from equality is highly significant ($\chi^2 = 19{\cdot}4$; with 1 D.F., $P < 0{\cdot}001$), so that in terms of the *numbers* of inclusions revealed, the FA method holds a considerable advantage.



Text-fig. 1. Frequency distribution of numbers of inclusions in duplicate thin smears stained by iodine or by FA.

Failure of inclusions to stain with FA

Comparative inclusion counts were made on slides stained first by FA and then by Giemsa. The second count was usually the higher, sometimes by as much as twofold; and in a few of our many paired photographs it was apparent that some typical inclusions that stained with Giemsa had not previously stained with FA (Plate 1*e*, *f*).

Sequential staining with FA and Giemsa

Efforts were made in the early part of these researches to verify by subsequent Giemsa staining all inclusions that were detected by the FA method. At that time, slides were stained immediately after fixation, and the quality of subsequent Giemsa staining was good. Later on, slides were stored for some time before FA staining, and both cells and inclusions stained poorly with Giemsa. Table 9 shows the effects of varying the time and temperature of storage on replicate slides from two patients. The ability to stain with FA was best preserved at 4° C. or lower; at −60°C. (not shown in the table) it was unimpaired after 3 years. By contrast, subsequent Giemsa staining was best in preparations held at room temperature. For stored slides the most satisfactory results were eventually obtained with the following procedure, to which unsuccessful attempts to follow the FA stain with

iodine made an unexpected contribution. The unfixed scrapings were stored at −60° C. For FA staining they were thawed, fixed at room temperature in acetone for 10 min. followed by 5% formalin for 2 min., rinsed quickly in PBS and air-dried. After FA staining, the smears were rinsed well in 5% formalin, treated for 10 min. in ammoniated iodine, immersed in methyl alcohol for 30 min., and then stained with Giemsa/May–Grünwald; the best results were obtained by omitting the customary post-stain alcohol rinse.

Table 9. *The influence of time and temperature of storage on the sequential staining of conjunctival scrapings with FA and Giemsa*

Time stored (days)	Patient and eye	Temperature of storage: Room FA*	Room G†	4° FA	4° G	−30° FA	−30° G
1	11 R	3	3	ND		ND	
	11 L	3	3				
	13 R	3	3				
	13 L	3	3				
5	11 R	2	3	3	2	ND	
	11 L	0	0	3	2		
	13 R	2	3	3	2		
	13 L	2	3	3	2		
10	11 R	1	3	3	2	3	1
	11 L	1	3	3	2	3	1
	13 R	2	3	3	2	3	1
	13 L	1	3	3	2	3	1
15	11 R	0	3	3	2	ND	
	11 L	0	3	3	2		
	13 R	0	3	3	2		
	13 L	0	3	3	2		
30	11 R	0	3	3	2	3	1
	11 L	0	3	3	2	3	1
	13 R	0	3	3	2	3	1
	13 L	0	3	3	2	3	1

ND, not done.

* FA stain: inclusions not seen (0), faint (1), moderately bright (2), very bright (3).

† Giemsa/May–Grünwald stain: scored from 0 (cell architecture lost, inclusions unrecognizable) to 3 (cells and inclusions well stained).

DISCUSSION

Before comparing the efficacy of the iodine stain with that of Giemsa, a discussion of certain technical considerations may be of interest.

The advocates of the FA method rightly claim that it has the advantage of serological specificity; on the other hand, the morphology of an iodine-stained inclusion is so characteristic that it can be identified with almost no possibility of error. This statement is based on many years' experience of the confirmation of iodine-stained inclusions by subsequent staining with Giemsa.

We may here refer again to the difficulty – which appears to be a common

experience – of verifying the morphology of inclusions with Giemsa after they have been stained with FA. Storage of the slides at subzero temperatures and their treatment with serum during FA staining both seem to impair the quality of subsequent Giemsa staining. Conversely, occasional inclusions failed to stain with FA, but were proved by serial photography to stain with Giemsa applied afterwards. Perhaps the most likely explanation is that penetration of antiserum or conjugate into the cell was prevented by a layer of mucus or other substance. The heterogeneous reactions of unabsorbed anti-MRC-4*s* sera make it improbable that the failure to stain some inclusions is attributable to infections with mixtures of serotypes, which in any event appear to be very rare (Bell, McComb, Nichols & Roca-Garcia, 1970). For the same reason, it is unlikely that false negatives resulted from the use of a serum prepared against a type 2 TRIC agent. The antiserum to MRC-4*s* grown in BHK-21 cells stained antigens representing the main trachoma serotypes 1, 1b and 2 identified by the Harvard/ARAMCO workers (McComb & Bell, 1967), and serotypes D, E and F that consist mostly of strains isolated from ocular and genital tract syndromes other than trachoma (Alexander, Wang & Grayston, 1967). In this connexion it is interesting to note that in an investigation still in progress, 21 of 52 trachoma agents isolated in Gambian villages were type 1 and 31 were type 2; no type 1b strains were encountered. In Bathurst, the capital of The Gambia, type F agents were isolated from the eye of an infant with neonatal TRIC conjunctivitis, and from the urethra of the father (Sowa, Sowa & Collier, 1968; Collier, Sowa & Sowa, 1969).

We had supposed that the ability to examine much thicker smears with iodine than is possible with FA would increase the chance of finding inclusions; but the comparison of thick and thin iodine-stained smears in study no. 1 suggested that this does not hold true, at least in terms of the number of positive diagnoses.

In considering our findings, the important point must be made that results obtained with the iodine stain are greatly influenced by the technique employed. Hanna and co-workers (1965) found that in 13 experimentally infected volunteers, TRIC inclusions were demonstrated in 11 by FA, in 3 by Giemsa and in none by iodine; but in this investigation the iodine-stained slides were wet-mounted (L. Hanna, personal communication). For best results, it is essential to employ dry preparations; depending on the optical properties of the microscope used, it may be advantageous to cover the smear with a thin coating of immersion oil.

It is apparent that the FA and iodine techniques for staining TRIC inclusions in the conjunctiva each have their advantages and disadvantages. In study no. 1, both methods yielded the same proportion of inclusion-positive specimens; in study no. 2 the proportion demonstrated as positive by FA was higher, but not significantly so. There was, however, a very significant increase in the numbers of inclusions revealed by FA as compared with iodine, probably because antibody stains inclusions at all stages of their formation, whereas iodine stains only mature inclusions that possess a carbohydrate matrix.

The skew distribution of the numbers of inclusions (Text-fig. 1) deserves comment. The frequency rapidly diminished from a peak value of 1–5 inclusions per scraping, and there were few instances of specimens containing from 21–50 inclu-

sions. As determined by the FA test, however, there was a fair number of scrapings with counts of more than 50 inclusions; in some instances, several hundred were present. The finding that certain children are liable to produce large numbers of inclusions recalls observations in artificially infected baboons; some of these animals – not necessarily those with the most severe inflammatory response – respond similarly to infection with TRIC agent (Collier, 1967).

Our conclusions are that where good laboratory facilities are available, FA staining is somewhat preferable to iodine for demonstrating conjunctival inclusion bodies. It should, however, be remembered that the technique demands comparatively elaborate reagents that must be carefully prepared, a first-rate fluorescence microscope, and observers with a high standard of training. Iodine-staining can be done with simple reagents and equipment, is much less time-consuming, and requires less in the way of skilled personnel. Since slides can even be stained and examined in villages, the method is valuable when large numbers of scrapings have to be screened under primitive conditions. It is unlikely that the proportion of positive diagnoses will be much less that with the FA method.

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EXPLANATION OF PLATE

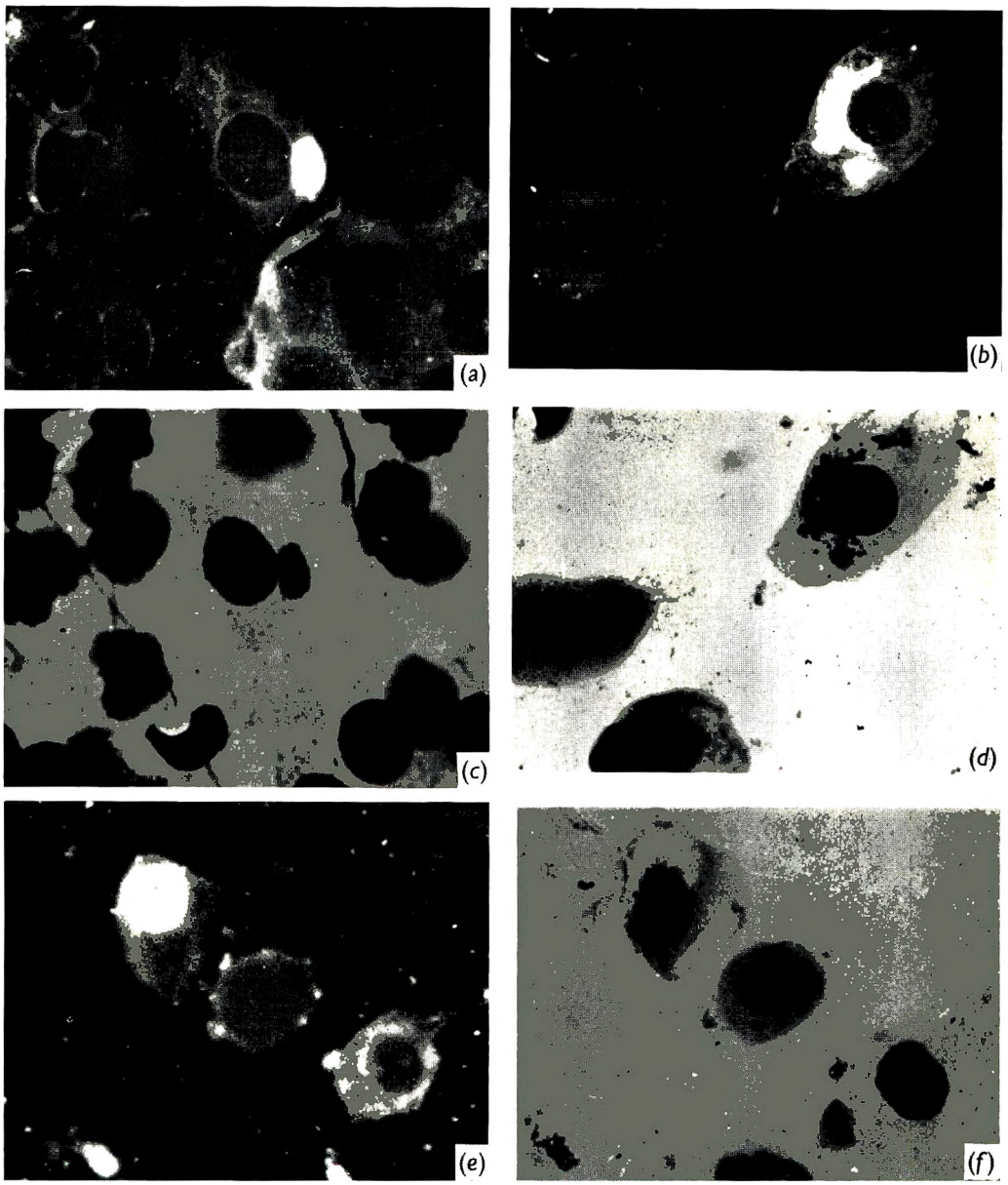
Conjunctival scrapings from trachoma patients, × 2000.

(*a*, *b*) Inclusion bodies within epithelial cells, stained with fluorescent antibody (FA). Bright field illumination; the dark background is due to the filter combination used.

(*c*, *d*) The same cells stained subsequently with Giemsa/May–Grünwald.

(*e*) Staining and illumination as (*a*), (*b*).

(*f*) The same field stained with Giemsa/May–Grünwald; three inclusions that did not stain with FA are now visible in the middle epithelial cell.


(a)
(b)
(c)
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(e)
(f)

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Sensitivity of trachoma agent to streptomycin and related antibiotics*

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SUMMARY

For isolating trachoma (TRIC) agent from conjunctival scrapings, both streptomycin and neomycin were effective in preventing bacterial contamination, but at high concentrations neomycin was rather more inhibitory to TRIC agent. Prolonging storage of scrapings with neomycin at 4° from 30 min. to 24 hr. reduced the bacterial contamination rate, but also diminished the chance of isolating TRIC agent at the 1st passage. Two freshly isolated TRIC agents differed in their susceptibility to neomycin and streptomycin. Kanamycin and framycetin appeared to be less suitable than streptomycin for use in isolation of TRIC agent. In isolation studies, the possibility of inhibiting TRIC agent by high concentrations of antibotics, including streptomycin, should be borne in mind.

INTRODUCTION

Trachoma is so frequently associated with bacterial infection of the conjunctiva that isolation of its aetiological agent was possible only by using streptomycin (T'ang, Chang, Huang & Wang, 1957; Collier & Sowa, 1958; Sowa & Collier, 1960), neomycin (Sowa, Sowa, Collier & Blyth, 1965) and other antibiotics singly or in combination to inhibit bacterial contaminants (e.g. Collier, Duke-Elder & Jones, 1960; Hanna *et al.* 1962; Holt *et al.* 1967). These drugs did not seem to inhibit trachoma/inclusion conjunctivitis (TRIC) agents propagated in yolk sac; but the comparatively low isolation rates often reported suggested the possibility that some failures at least were due to inhibition of TRIC agents by drugs to which they were supposedly completely resistant. The investigation described here was undertaken to test this assumption and to determine whether other antibiotics related to streptomycin would be more suitable for isolating TRIC agents.

MATERIALS AND METHODS

Diluent. Sucrose potassium glutamate (SPG) was used as diluent (Bovarnick, Miller & Snyder, 1950). It was sterilized by autoclaving at 5 lb./in.^{2} pressure for 10 min.

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Antibiotics were obtained as sterile powders for injection and were dissolved in diluent. Streptomycin sulphate (Streptomycin); Diston Products Ltd.; potency 740,000 μg./g. Neomycin sulphate (Mycifradin); Upjohn Ltd. potency 700,000 μg./g. Framycetin sulphate (Framygen); Genatosan Division of Fison Pharmaceuticals Ltd.; potency not stated. Kanamycin sulphate (Kantrex); Bristol Laboratories Ltd.; potency not stated.

Conjunctival scrapings from children with active trachoma were examined for the presence of inclusions by the ammoniated iodine method of Sowa *et al.* (1965). For isolating TRIC agent pooled scrapings from both eyes were collected into 2 ml. diluent, disrupted in a mechanical homogenizer (Measuring and Scientific Equipment Co. Ltd.) at full speed for 1 min. and divided into 4 aliquots. To each, 0·5 ml. of the appropriate antibiotic solution was added after which it was kept at 4° C. for 30 min. In comparative experiments some specimens with neomycin were tested after storage at 4° C. for 24 hr.

Each sample was inoculated into three embryos; three blind passages were made before any result was accepted as negative.

Chick embryo inoculation. Fresh hens' eggs were collected in villages, washed briefly in soapy water, rinsed quickly in running tap water and dried on a clean towel. They were incubated at 37° C. and 50% relative humidity with one daily turning, and were candled at 3 days and again just before inoculation on the 6th day. The embryos were inoculated by the yolk-sac route and were incubated at 35° C. and 50% humidity. After inoculation they were first candled at 48 hr., when dead embryos were discarded, and thereafter daily until the 12th or 14th day, depending on the experiment. All yolk sacs harvested after 48 hr., including those of survivors, were examined for elementary bodies by staining smears with Giemsa/May–Grünwald.

For passage the entire yolk sac was placed in a screw-cap bottle with an equal weight of SPG. After homogenization as described above, the suspension was inoculated into the next set of eggs immediately, or after storage at $-60°$ C.

Suspensions of TRIC agent for use in testing antibiotics. Partly purified stocks were prepared from two recently isolated strains and from an earlier stored strain, all in their third egg passage. Their full designations (Gear, Gordon, Jones & Bell, 1963) are WAG/MRC-51/OT, WAG/MRC-84/OT and WAG/MRC-60/OT. They are referred to in the text by their MRC numbers only. These strains were grown in the presence of the concentration of the antibiotic with which they were originally isolated. Chick embryos were harvested on the day of death. After shaking each yolk sac by hand in 10 ml. SPG the membranes were discarded; the fluids were pooled and centrifuged for 10 min. at 200***g*** at 10° C. The deposit was discarded and the supernatant was centrifuged at 4500***g*** for 1 hr. at 10° C.; the deposit containing the TRIC agent was resuspended in SPG to give half the volume of the original suspension, i.e. about 20% dilution of the yolk sac. The stock suspensions were stored at $-60°$ C. in 1 ml. amounts. When required they were thawed rapidly in a water bath at 37° C. and suitably diluted.

Infectivity titrations. The suspensions were titrated without antibiotic by inoculating tenfold dilutions into groups of 10–12 embryos which were then incu-

bated for 11 days. The 50% infective dose (EID 50) was calculated by the method of Reed & Muench (1938).

Single dilution titrations. Inocula were prepared by diluting the stock suspension 1/100. Samples treated with a 'high' concentration of an antibiotic (see under 'Results') for 30 min. at 4° C. and controls without antibiotic were inoculated into large groups of embryos, usually about 30 in number. They were candled every 12 hr. for 14 days. In calculating the average day of death embryos still alive on day 14 were counted as dying on day 15.

Assays of antibiotic activity. Tenfold dilutions of each drug were each mixed with a 1/100 suspension of MRC-60 and inoculated immediately into groups of about 12 embryos. The eggs were incubated for 12 days thereafter; embryos surviving for this time were counted as dead on day 13. The index of protection was calculated according to the following formula, which allows for embryos surviving uninfected for the maximum period:

$$\text{index of protection} = \frac{MS_t - MS_c}{MS_m - MS_c} \times 100,$$

where MS_t, MS_c are the mean survival times of the test and control embryos respectively and MS_m is the maximum possible survival time after inoculation (13 days in these tests).

RESULTS

Isolation of TRIC agent from conjunctival scrapings

Influence of neomycin and streptomycin

The amount of TRIC agent present in conjunctival scrapings was insufficient to permit titrations of sensitivity to antibiotics. In consequence sensitivity was assessed by comparing isolation results from replicate samples of conjunctival

Table 1. *Isolations of TRIC agent from replicate scrapings inoculated into chick embryos together with high and low concentrations of streptomycin and neomycin*

Concentration of antibiotic (μg./egg)	No. of replicate specimens	Specimens positive at passage no. 1	2	3	Total no. of isolations	Isolation failures due to bacterial contamination	Isolation rate
Neomycin							
11,250	83	21	6	2	29	2	29/81 (35·8%)
112	83	31	2	0	33	4	33/79 (41·7%)
Streptomycin							
10,713	39*	15	2	0	17	0	17/39 (43·6%)
107	39*	17	0	0	17	1	17/38 (44·6%)

* Common to the neomycin series.

scrapings to which either 'high' or 'low' concentrations of the antibiotics were added. To assure even dispersion each specimen was homogenized for 1 min.; infectivity was not increased by extending this time. The 'high' concentrations

of neomycin (11250 μg./egg) and of streptomycin (10713 μg./egg) were the greatest that could be obtained; the 'low' concentrations were 1/100 dilutions of the high doses.

Thirty-nine specimens were common to the streptomycin and the neomycin series; another 44 were tested in the presence of neomycin alone. At the high concentration of neomycin, which none the less was insufficient to eliminate all bacterial contamination, there were four fewer isolations than from replicate specimens treated with the low dose of the drug. Streptomycin under similar conditions was less detrimental. The inhibitory effect was also evident from the delayed appearance of the TRIC agent, sometimes until the second or third embryo passage (Table 1).

Table 2. *The effect of neomycin and streptomycin on infection of the individual embryos with TRIC agent at the first passage*

Concentration of antibiotic (μg./egg)	No. of embryos inoculated with 'potentially positive' specimens*	No. of valid inoculations†	Embryos infected with TRIC agent
Neomycin			
11,250	39 × 3 = 117	107	37 (34·6 %)
112	39 × 3 = 117	95	61 (64·2 %)
Streptomycin			
10,713	19 × 3 = 57	53	32 (60·4 %)
107	19 × 3 = 57	51	35 (68·6 %)

* I.e. from which TRIC agent was isolated at any concentration of either antibiotic.
† I.e. excluding deaths due to non-specific causes or bacterial contamination.

Table 3. *Isolation of TRIC agent from inclusion-positive and inclusion-negative specimens in the presence of neomycin and streptomycin*

Concentration of antibiotic (μg./egg)	Nos. of specimens: Total	Isolation-positive	Contaminated	Isolation rate
	(*a*) Inclusion-positive patients			
Neomycin				
11,250	17	14	1	14/16 (87·5 %)
112	17	15	1	15/16 (93·8 %)
Streptomycin				
10,713	6	6	0	6/6 (100·0 %)
107	6	5	1	5/5 (100·0 %)
	(*b*) Inclusion-negative patients			
Neomycin				
11,250	22	15	1	15/21 (71·5 %)
112	22	18	3	18/19 (94·7 %)
Streptomycin				
10,713	13	11	0	11/13 (84·6 %)
107	13	12	0	12/13 (92·3 %)

Decrease in number of positive embryos in the primary passage. The figures in Table 2 are based only on embryos inoculated with 'potentially positive' specimens, i.e. those from which TRIC agent was isolated at any concentration of either antibiotic. With the low concentration of neomycin nearly twice as many embryos became infected as with the high; with both high and low concentrations of streptomycin the percentages of positive chick embryos were similar to that obtained with the lower dose of neomycin (Table 2).

Table 4. *Isolation of TRIC agent in embryonated eggs after storage of the scrapings at 4° C. in the presence of neomoycin*

Concentration of neomycin (μg./egg)	No. of replicate specimens	Exposure to neomycin at 4° C. before inoculation (hr.)	No. of isolations: At passage 1	2	3	Spoilt by contamination	Isolation rate
11,250	44	0·5	14	1	2	1	17/43 (39·6 %)
112	44	0·5	18	0	0	2	18/42 (43·8 %)
11,250	44	24·0	10	2	3	0	15/44 (34·1 %)
112	44	24·0	13	3	0	0	16/44 (36·4 %)

Isolation rates from inclusion-positive and inclusion-negative patients. From inclusion-positive patients two isolations were lost at high and one at low concentrations of neomycin (not counting losses from bacterial contamination). Only one isolation was lost, and this by contamination, from material treated with streptomycin. The inhibitory effect of both antibiotics on TRIC agent was somewhat higher, but not significantly so, in the inclusion-negative series (Table 3).

Treatment of scrapings with neomycin at 4° C. The scrapings used in the experiments described above were obtained from patients at the laboratory; in field work it is often necessary to store them on ice from 6 to 24 hr. before inoculating chick embryos. These conditions were simulated by comparing the isolation rates from specimens left in contact with two concentrations of neomycin for 30 min. with that from replicate scrapings stored for 24 hr. at 4° C. It was impracticable to test for isolations without any antibiotic on account of bacteria which are always present in these specimens. With either concentration of neomycin prolonging storage from 30 min. to 24 hr. diminished the chance of isolating TRIC agent in the first passage, but the influence of storage is less pronounced if subsequent passages are taken into account (Table 4). With specimens stored for 30 min., three attempts at isolation were spoiled by bacterial contamination, whereas none were spoiled with those stored for 24 hr.

Inhibition by antibiotics of TRIC agent in ovo

Prolongation of mean survival times by neomycin and streptomycin. Strain MRC-51 was isolated only at the 'low' concentration of streptomycin but not with the 'high' concentration, and with neither concentration of neomycin; likewise, strain MRC-84 was isolated only with the lower concentration of neomycin and with neither 'low' nor 'high' concentrations of streptomycin. To test the assumption

that failure to isolate them at the higher concentrations of these drugs was due to unusually pronounced sensitivity, they were titrated in eggs with the 'high' concentrations by the single dilution method. Four replicate determinations were made with each antibiotic, and four without, for each strain. Fig. 1 compares the results with serial dilution titrations of the two strains without antibiotic. The effects of neomycin and streptomycin were similar and are not shown separately; the vertical bars show the ranges of results obtained in the single dilution titrations. The prolongation of survival time afforded by these antibiotics to embryos inoculated with MRC-84 was comparable to that obtained by diluting the inoculum a hundred-fold; the effect of these drugs on MRC-51 was much less.

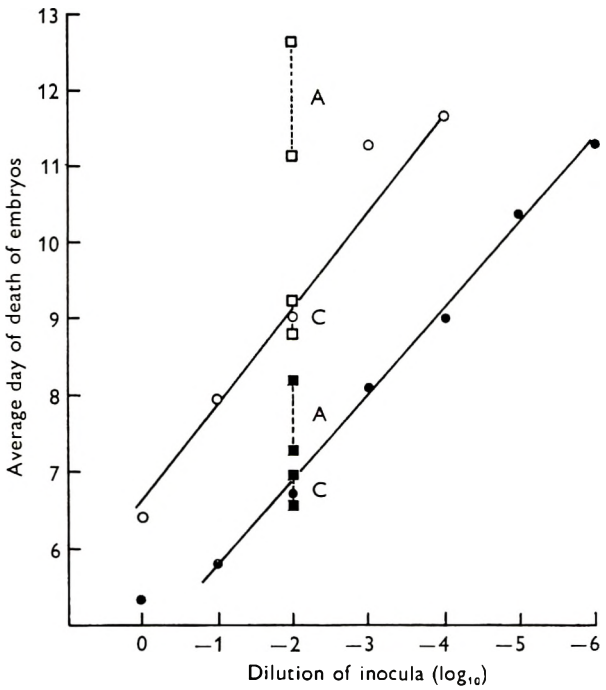

Fig. 1. Dose–response curves of MRC-51 ($10^{4\cdot6}$ EID50 undiluted) and MRC-84 ($10^{4\cdot3}$ EID50 undiluted), compared with single dilution titrations in the presence of high concentrations of neomycin or streptomycin. Closed symbols, MRC-51; open symbols, MRC-84; circles, dose–response curves. Squares, single dilution titrations; A, with antibiotics; C, controls. The dotted vertical bars represent the ranges of four separate determinations for each antibiotic, and four control tests without antibiotic.

The effect of framycetin and kanamycin. Isolation of TRIC agents would be facilitated by the use of an antibiotic to which they were completely resistant, but which was active against a wide range of bacteria; with this in mind framycetin and kanamycin were compared with streptomycin and neomycin, to which they are related. For this experiment, strain MRC-60 was used; it was isolated in the presence of neomycin, 700 μg. per embryo. Fig. 2 shows that both kanamycin and framycetin were more inhibitory than neomycin and streptomycin. From this

point of view, the two last-named drugs appear to be the more suitable for use in isolating TRIC agents; but in preliminary experiments neither of them in a dose of 160 μg. per embryo protected against a 'streptomycin-sensitive' staphylococcus injected at the same time. Low doses of these antibiotics are thus unlikely to suppress bacterial contamination effectively.

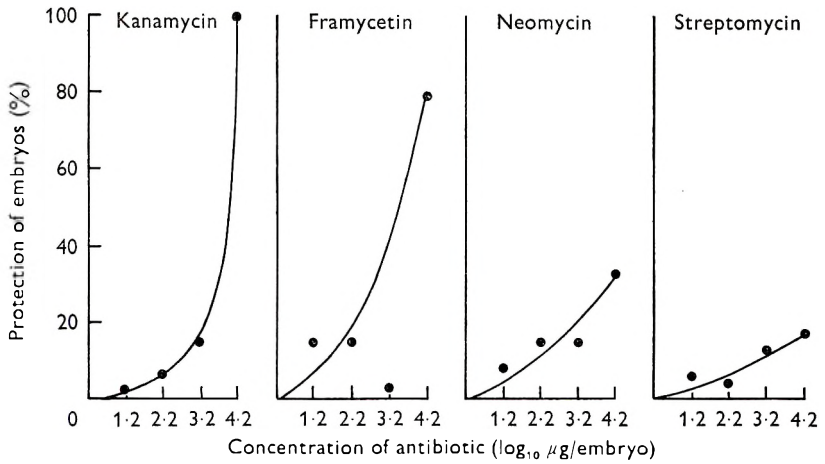

Fig. 2. Dose–response curves representing protection of chick embryos inoculated with $10^{3\cdot2}$ EID 50 of TRIC agent MRC-60. At the $10^{3\cdot2}$ μg dose of framycetin, 8/10 embryos died within 2 days, probably because of bacterial contamination.

DISCUSSION

T'ang and co-workers (1957) were the first to point out the 'viricidal' action of several drugs on TRIC agent in egg culture. A notable exception was streptomycin, which they found 'frankly negative'; other workers reached the same conclusion and high concentrations of this drug alone were often used to assist isolation (Collier & Sowa, 1958; Grayston *et al.* 1960; Murray, Guerra, Abbot & McComb, 1962). Bacterial contamination was still troublesome however, and neomycin (Sowa *et al.* 1965) or other antibiotics in various combinations were used (e.g. Holt *et al.* 1967). Streptomycin, which it was generally agreed did not inhibit TRIC agent in embryonate eggs, reduced the number of inclusions formed in chick entodermal culture (Gordon & Quan, 1962). As the embryonate egg still remains in general use for isolating TRIC agents from natural and experimental infections and the success rate is often comparatively low, an improvement of the method would be of considerable value.

The inoculum, consisting of conjunctival epithelium, inflammatory cells, tears and mucus usually contains a variety of bacteria that multiply in the embryo more rapidly than TRIC agent, and as both may be affected by antibiotics, the TRIC agent isolation success rate will depend on differences in sensitivity. Tarizzo & Nabli (1963) tested a number of antibiotics against a variety of TRIC agents, all of which were relatively insusceptible to neomycin, framycetin and kanamycin; however, our experience suggests that none of these drugs was more suitable than

streptomycin, and even this should be used in moderate concentration and not added until the time of inoculation. By way of practical recommendation we may here refer to a recent study (unpublished) in which 53 scrapings from trachoma patients were cultured in the presence of streptomycin 400 μg./egg. The rate of isolation was 70% and only two attempts failed because of bacterial contamination; this compares favourably with our best results in the past (Sowa *et al.* 1965).

We are grateful to Dr Shiona Sowa for undertaking the clinical examinations and for collecting the conjunctival scrapings; and to Professor L. H. Collier for his help in preparing this paper for publication. We also thank Dr I. A. McGregor and his staff at the Medical Research Council Laboratories, Fajara, The Gambia for various facilities.

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Criteria for measuring the efficacy of trachoma vaccines in baboons

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SUMMARY

Trachoma vaccines are usually assayed by testing their ability to protect monkeys or baboons against subsequent challenge of the conjunctiva with a pathogenic strain of trachoma/inclusion conjunctivitis (TRIC) agent. In such experiments the course of infection in vaccinated baboons was compared in terms of arbitrary scores assigned to a range of clinical signs, and of counts of TRIC inclusions in conjunctival scrapings. Analysis of many such scores indicated that after a large challenge dose of strain MRC-4*s*, the scores for signs of inflammation reached their maximum earlier than the follicle score; the inflammation score was closely related to the number of inclusions, whereas the follicle score was not. With this system, the optimum periods for eliciting differences between vaccinated and control measures varied according to the sign used; it was later for follicles than for inflammation or inclusions. For assessing the influence of vaccination, the mean of the inflammation scores read weekly for the first 3 weeks after challenge and the mean inclusion score over the same period were equally satisfactory, and either was rather better than the mean of three follicle scores taken over the period 3–6 weeks.

For assessing the influence of vaccines or therapeutic agents on experimental trachoma it is important to determine which signs discriminate best between treated and control animals, and the optimum times for measuring them.

INTRODUCTION

The potency of trachoma vaccines is usually assessed in terms of their ability to protect against infection with a pathogenic trachoma or inclusion conjunctivitis (TRIC) agent. To do this, the challenge dose is inoculated into the conjunctivae of vaccinated monkeys or baboons, and the course of infection is compared with that in an unvaccinated control group. It is obviously desirable, first, to be able to express the severity of infection quantitatively, and second, to determine which features are most useful for estimating the influence of immunization.

In man, the main conjunctival and corneal signs of trachoma were long ago assigned scores for severity, first as 0, +, ++, +++, and later as numbers (W.H.O. Expert Committee on Trachoma, 1962). In 1966 a W.H.O. Scientific Group on Trachoma Research recommended a much more comprehensive system primarily for use in field studies. Soon after their start in the early 1960s it became apparent that a scoring system was also needed for vaccination experiments in non-human primates; but because corneal lesions and scarring do not occur in simians except in special circumstances, it did not have to be as elaborate as for man. In 1960, Grayston and co-workers assessed the potency of vaccines in monkeys solely on the presence or absence of conjunctival follicles. Collier (1961) working with baboons assigned separate scores to signs of inflammation (comprising conjunctival hyperaemia, oedema and infiltration) and to conjunctival follicles. Dawson, Jawetz, Thygeson & Hanna (1961) recorded the intensity of hyperaemia, discharge, infiltrate and conjunctival follicles; on the basis of specificity and reproducibility of assessment they considered that follicles were the most suitable lesions for scoring, and used such scores to compare the infections induced by different TRIC agents and to assess the influence of vaccination. Mordhorst (1967) later extended this system to include six more physical signs, the proportion of inflammatory cells in conjunctival scrapings and the number of inclusion bodies. Wang (1967) assigned numerical scores to a range of physical signs in monkeys and used them to compare the infections induced by different TRIC agents and to assess the influence of vaccines and penicillin treatment. Collier & Blyth (1966*a*) were the first to employ statistical techniques; they described a scoring system for infection in baboons, and used the values in analyses of variance to determine the significance of differences in the responses to challenge of vaccinated and control animals. The desirability of introducing statistical methods was emphasized by the wide ranges of the 95% confidence limits on scores for individual vaccinated animals.

From what has already been said, there is obviously much variation in the criteria employed somewhat arbitrarily by different workers for measuring the severity of infection. Those used so far fall into three categories: clinical signs, appearance in conjunctival scrapings of inflammatory cells, and inclusion bodies. Of these, only the presence of inclusions is pathognomonic, and it therefore serves as a base line to which other variables can be related. With regard to the clinical appearances, Collier (1967) pointed out that because follicular hyperplasia is the dominant lesion it has been used by some as the sole index of severity of infection; but since these lymphoid follicles are probably part of the antibody-forming apparatus they could be regarded as secondary lesions that may not provide such a direct indication of severity as do the signs of inflammation – lid oedema, discharge, conjunctival infiltration and hyperaemia – which reach their maximum earlier. This supposition was borne out by the results of examining 41 unvaccinated baboons 4 times at weekly intervals after conjunctival inoculation with the MRC-4*s* strain of TRIC agent. There was a highly significant positive correlation between the numbers of inclusion bodies and the cumulative scores for signs of inflammation, but none between the inclusion counts and scores for follicles. The

present paper describes the further analysis of these and other data, and in particular the value of various criteria for assessing the degree of immunity induced by trachoma vaccines.

MATERIALS AND METHODS

Immunization experiments in baboons

Part of the data to be analysed are derived from five experiments in which groups of young baboons (*Papio cynocephalus*) were immunized parenterally with live TRIC agents. The infection induced by inoculating a large dose of TRIC agent into one eye was compared with that in non-immunized control animals. The challenge strain was originally known as LB4; its full designation in the 'Montreal system' (Gear, Gordon, Jones & Bell, 1963) is TRIC/2/GB/MRC-4/ON. In these experiments, only the 'slow-killing' parent strain MRC-4*s* (Reeve & Taverne, 1963, 1967) was used for challenge.

These five immunization experiments have been published with the following reference numbers: no. 2 (Collier & Blyth, 1966*a*); nos. 8 and 10 (Collier & Blyth, 1966*b*); nos. I and II (Collier & Smith, 1967). In all of them, the vaccines used exerted a greater or less measure of protection as tested by analysis of variance and by determining whether the scores for individual vaccinated animals fell within the confidence limits computed for the experiment in question. In the present paper the data for unvaccinated animals are supplemented with the results of challenging control baboons with MRC-4*s* in five other experiments in which the vaccines failed to immunize.

The methods of inoculating and examining baboons and of staining inclusions by the iodine method were described by Collier (1961) and Collier & Blyth (1966*a*). Each animal was examined on the day of challenge (day 0) and in most instances on days 7, 14, 21, 28 and 42 thereafter. All examinations were made by one person (L.H.C) without reference to previous findings.

In some experiments the intervals between examinations were somewhat different, so that each of the five examinations undergone by every animal is referred to in terms of a range of days after challenge, e.g. 4–8, 9–15.

Scoring system

The inflammation score is the sum of the scores for (*a*) external oedema of the lids, (*b*) purulent discharge, (*c*) conjunctival hyperaemia and (*d*) conjunctival infiltration (recognized by loss of transparency, oedema and thickening). Each of these signs was scored 1, 2 or 3 according to severity, 0 if absent. Hyperaemia and infiltration were scored separately for the upper and lower lids.

The follicle score is the sum of those for the upper and lower lids using the same scale as for inflammation. The small superficial translucent follicles characteristic of 'non-specific folliculosis' were sometimes present in normal animals and were ignored.

Occasionally one or another lesion was present in the inoculated eye on the day of challenge, or appeared in the control eye during the course of observation.

Rules for correcting scores appropriately were based on two assumptions: (*a*) that physical signs in the control eye appearing or increasing at any time after the day of inoculation (day 0) were caused by inadvertent contamination at the time of inoculation or by subsequent cross-infection from the inoculated eye. From this it follows that scores for the control eye were subtracted from the test eye scores only when the lesion was present on day 0. (*b*) That day 0 scores would remain stationary over the comparatively short period of observation.

The rules are:

(1) If a score for a given physical sign was recorded for the control eye on day 0, this and all subsequent control scores (if any) were subtracted from the corresponding score for the inoculated eye.

(2) If the control eye score was greater than the inoculated eye score, the latter was assigned zero value (because a negative value means 'better than normal', which is unrealistic).

(3) All control eye scores not covered by (1) were ignored.

(4) If a physical sign was present in the inoculated eye on day 0, the score was subtracted from all subsequent corresponding scores for that sign, subject to rule (2).

The inclusion score was derived from the total number of inclusions in scrapings from both upper and lower lids of the inoculated eye. Because the distribution of the numbers of inclusions was decidedly skew a transformation of the number was used as the score, namely $\log_{10}$ (number of inclusions + 1). To avoid counting large numbers of inclusions, slides containing more than 30 were recorded simply as '> 30', but for statistical analysis a value of 32 was arbitrarily assigned to such readings.

RESULTS

Time course of infection in control and vaccinated baboons

Fig. 1 gives the mean values of the scores at various intervals after challenge with TRIC agent. In the 58 unvaccinated control animals the scores for inflammation and inclusions both rose rapidly, remained high for the first 3 weeks, and then diminished. By contrast, the mean follicle score rose steadily to a maximum at 24–34 days, and remained high until at least the 6th–7th week. In vaccinated animals, the inflammation and follicle scores followed the same trends as in the controls, but with lower values. The scores for inclusions behaved rather differently however; the highest score in vaccinated animals was attained within the first week, and after the 2nd week it declined to a very low value. In other words, vaccination appeared to change the shape of the inclusion score curve from concave downwards to concave upwards, whereas for the other two variables the shape remained much the same.

These findings suggest that the best indicators of the effect of vaccination would be the average score for inflammation over the period 4–22 days, or for follicles over the period 17–49 days, since the differences between the scores for vaccinated and control animals were greatest at these times. Although the shape of the inclusion curve in vaccinated animals differed from that in the controls, the average

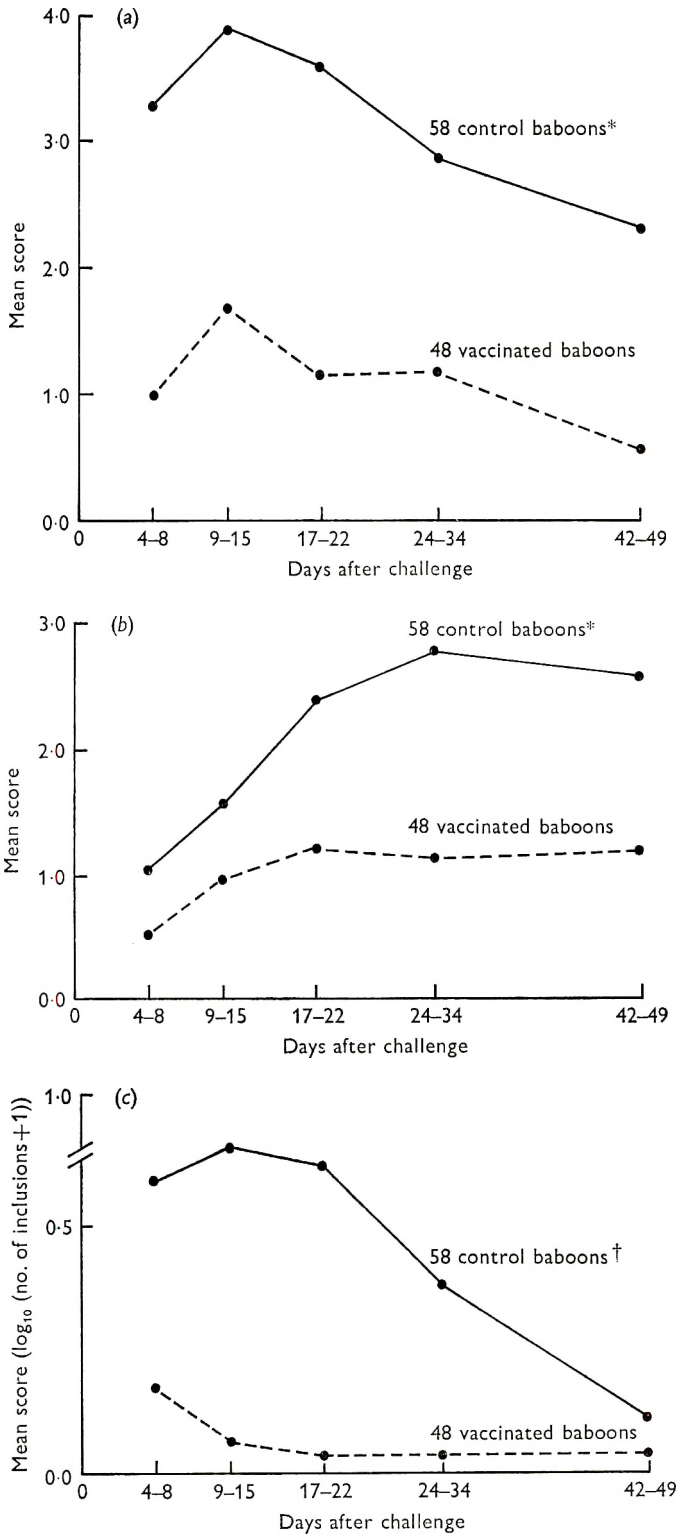

Fig. 1. Mean values of scores at various intervals after challenge of baboons with TRIC agent. The abscissae are marked at the midpoints of the periods indicated. (*a*) Scores for inflammation. (*b*) Scores for follicles. (c) Scores for inclusions. * Value at 42–49 days based on 45 animals only. † Value at 4–8 days based on 57 animals and at 42–49 days on 39 animals only.

score for inclusions over the period 4–22 days appeared to be the most sensitive indicator of the effect of vaccination.

Interrelationships of the variables in unvaccinated animals

Before comparing inflammation, follicles and inclusions in terms of the effects of vaccination upon them, their interrelationships in all 58 unvaccinated animals

Table 1. *Correlations between scores for inflammation (mean of readings at 4–22 days) follicles (mean of readings at 17–49 days) and inclusions (mean of readings at 4–22 days)* for 58 *unvaccinated baboons*

Variables correlated	Correlation coefficient	Probability (P)
Inflammation × follicles	0·14	> 0·100
Inflammation × inclusions	0·67	< 0·001
Follicles × inclusions	0·19	> 0·100

Table 2. *Values of Student's* t *for differences in mean scores between vaccinated and unvaccinated baboons in individual experiments*

Experiment no.	No. of baboons		Criterion for assessing efficacy of vaccination		
	Vaccinated	Control	Inflammation score (mean from 4–22 days)	Follicle score (mean from 17–49 days)	Inclusion score (mean from 4–22 days)
2	12	6	4·23***	3·12**	4·04**
8	6	6	3·23**	0·69	2·66*
10	5	5	2·63*	0·62	2·08
I	11	6	3·21**	1·22	2·89*
II	14	6	3·90**	2·76*	6·25***
All experiments	48	29	7·39***	3·95***	7·64***

* Significant at 5 % level; ** 1 % level; *** 0·1 % level.

were examined. Table 1 shows that there was a highly significant correlation coefficient of 0·67 between the inflammation score and the inclusion score, which is perhaps not surprising since they behaved similarly with respect to time. There was, however, little or no correlation between the scores for inflammation and follicles nor between those for inclusions and follicles.

Criteria for assessing the effects of vaccination

Table 2 gives the values of Student's t for the differences in the 'best indicator' scores between vaccinated animals and their controls in the separate experiments and in all combined. In general, whenever one of the measures indicates a significant difference, the others also do. The values of t are smallest for the follicle scores, but there is little to choose between the inflammation and the inclusion scores. The values of t for all experiments combined show the same pattern.

For measuring the influence of vaccination it seems that the mean of the in-

flammation scores read at weekly intervals for the first 3 weeks after challenge with MRC-4*s*, and the mean inclusion score over the same period, are equally good; either is preferable to the best measure based on follicles, namely the mean of three follicle scores taken over the period 3–6 weeks.

DISCUSSION

The analysis reported here confirms a previous observation that the number of inclusions is closely related to the intensity of inflammation induced in the baboon eye by TRIC agent, but not to the degree of follicular hyperplasia, which attains its maximum later (Collier, 1967). It is therefore not surprising that modification of the clinical signs associated with inflammation is a rather better index of the efficacy of a vaccine than prevention of follicle formation. As we have seen, however, some workers attach more importance to follicles than to other lesions; for example, the W.H.O. Scientific Group (1966) recommended giving extra weighting to the scores for mature follicles in man. Again, Wang's system for scoring TRIC infections in monkeys is so constructed that follicles are likely to be given a greater weight than other lesions; furthermore, unless some follicles are present no score at all is given to signs of inflammation, apparently because they are 'not as specific as trachomatous follicles'. In considering whether a given feature of the disease should be scored, and if so, how it should be weighted, the purpose of the study must be considered; a system that is suitable for an epidemiological survey in man may be much less so for vaccine assays in monkeys. In this connexion it is worth recalling the distinction between the *relative intensity* of trachoma (the degree of activity in an individual case at a given time) and the *relative gravity* (the degree of disabling complications or of active lesions that will lead to such sequelae if untreated) (World Health Organization, 1962); these indices may have little in common in man (Assaad & Maxwell-Lyons, 1967) and even less in simians, in whom sequelae are very rare. For assessing relative gravity in man it may be quite justifiable to weight the score for follicles, because their extent may determine the degree of subsequent cicatrization (World Health Organization, 1962). By contrast, vaccine experiments in monkeys involve the assessment of something corresponding more closely to relative intensity, to which follicular lesions are perhaps less relevant.

Most observers, ourselves included, have relied on the clinical appearances and on the presence of specific inclusion bodies to delineate the course of TRIC infection in the eye. Mordhorst (1967) also assigned a numerical score to the proportion of inflammatory cells in conjunctival scrapings; we have not used this variable, but our own observations on large numbers of slides suggest that it may well be a very useful guide to the severity of infection, especially in the early stages. By analogy with the distinction between inflammatory and follicular lesions, it is likely that the proportion of polymorphonuclear leucocytes would be a more useful index than that of mononuclear cells, which tend to appear late.

One must decide not only what to measure but when to measure it. As a rule, scores are taken during the period when the infection is most severe. Dawson,

Mordhorst & Thygeson (1962) examined their monkeys 2 or 3 times a week, and summated the 3 highest scores in both eyes for each clinical sign recorded during the first 3 weeks after inoculation. Collier & Blyth (1966*a*) examined baboons 1, 2, 3, 4 and 6 weeks after inoculation. The cumulative score for the first 4 weeks only was used for interpreting the results, since by the 6th week spontaneous regression of physical signs had diminished the differences between control and vaccinated animals. Wang (1967) employed an average obtained by adding the scores at weekly examinations and dividing the sum by the number of weeks of observation, sometimes as many as 25. Our findings indicate that the optimum periods for taking scores may differ with the variable being measured. The addition of scores taken much outside these periods will obviously impair the sensitivity of discrimination between treated and control animals; and it follows that scores for the various signs of infection should not be combined unless their time courses are similar.

Strictly speaking, our findings and the conclusions from them apply only to baboons inoculated with large doses of strain MRC-4*s*; alteration of the experimental conditions, particularly by diminishing the dose of TRIC agent, might well result in a different pattern of infection. Nevertheless, other experiments not reported here suggest that the relationships between inflammatory lesions, follicles and inclusions are likely to be generally true of infections induced by other pathogenic TRIC agents, at least in baboons. We hope that this study has illustrated the importance of defining the course of infection in control animals; of being selective in choosing criteria for assaying the influence of vaccines and therapeutic drugs; and of using statistical techniques for these purposes.

We are highly indebted to Dr I. Sutherland for his advice on the methods of statistical analysis used in this paper.

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