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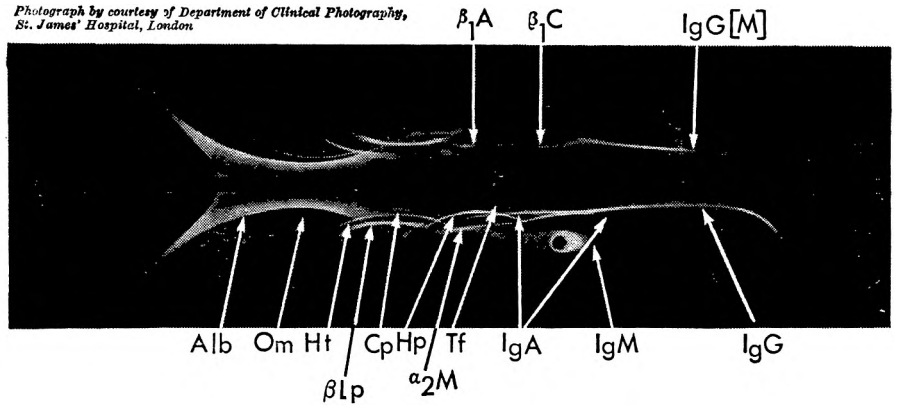

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Alb	Albumin	Cp	Ceruloplasmin
Om	Orosomucoid	Hp	Hemopexin
Ht	Haptoglobin	α_2M	α_2-macroglobulin
Lp	β-lipoprotein	Tf	Transferrin

IgA Immunoglobulin A (Syn: γ_1A; β_2A)
IgM Immunoglobulin M (Syn: γ-macroglobulin)
IgG Immunoglobulin G (Syn: γ_2; 7S γ)
IgG[M] Pathological IgG (Myelomatosis)
β_1A, β_1C } Subfractions of the lipoprotein

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Salmonella food poisoning associated with imported canned meat

By R. C. S. BURNETT
Assistant Medical Officer, City of Edinburgh
and B. I. DAVIES
Bacteriology Department, University of Edinburgh Medical School

(*Received* 30 *August* 1966)

A number of outbreaks of enteric fever and food poisoning have occurred in which canned foods have been incriminated, viz. the outbreaks of typhoid fever at Oswestry (Bradley, Evans & Taylor, 1951), Pickering (Couper, Newell & Payne, 1956), Harlow (Ash, McKendrick, Robertson & Hughes, 1964), South Shields and Bedford, and the outbreaks of staphylococcal food poisoning due to infected tins of canned peas (Bashford *et al.*, quoted by Tomlinson, 1965). The most recent outbreak of enteric fever attributed to canned meat was the outbreak in Aberdeen in the summer of 1964 (Report, 1965).

In October 1964 and in May 1965 two localized outbreaks of food poisoning, due to salmonella organisms, occurred in Edinburgh. In both outbreaks, epidemiological and bacteriological evidence pointed to imported canned meat as the source.

In the first outbreak the organism responsible was *Salmonella reading* and in the second *S. tennessee.*

The total number of persons affected was 94, 55 in the first outbreak and 39 in the second.

THE FIRST OUTBREAK

On Monday, 12 October 1964, the Public Health Department was informed that three persons, a father and his two sons, were to be admitted to the Edinburgh City Hospital as suspected cases of food poisoning. A common factor in their diet was chopped pork which had been sold on Saturday 10 October and eaten by all three on the evening of that day. The fourth member of the family, the mother, was unaffected. She had bought the chopped pork but did not eat any of it. Symptoms had started 8–11 hr. after ingestion of the chopped pork. A rectal swab was taken from the younger son on 11 October and was sent, together with the remains of the chopped pork left over from the meal, to the Bacteriology Department, Edinburgh University. A salmonella of group B, later shown to be *Salmonella reading*, was isolated from both the rectal swab and the chopped pork.

In view of these findings incriminating the chopped pork as the likely cause of the food poisoning, the shop from which the meat had been purchased was visited on the evening of 12 October. The owner of the shop volunteered the information that a customer's family had been affected the previous day with diarrhoea and

vomiting and that a shop assistant, who had been working at the cold meat counter on the morning that the suspected chopped pork had been on sale, was ill at home with 'gastric flu'.

Visits to these persons revealed that chopped pork had been eaten by all concerned and that the shop assistant had also eaten some boiled ham which had been on sale at the cold meat counter at the same time as the chopped pork.

By routine contact tracing, and notifications from the City Hospital, the University Laboratory and general practioners, who had been given information on the outbreak by letter, 55 cases of food poisoning, involving 21 families, were discovered.

Table 1. *Analysis of cases (first outbreak)*

	Total no. of infected cases	No. with symptoms	No. asymptomatic	No. admitted to hospital	No. of emergency admissions
Male	23	14	9	5	4
Female	32	20	12	5	2
Total	55	34	21	10	6

Table 2. *Food eaten by infected persons in first outbreak*

	No. eating C.P. only	No. eating B.H. only	No. eating C.P. and B.H.	No. eating neither
Male	11	4	3	5
Female	12	6	5	9
Total	23	10	8	14

C.P. = chopped pork; B.H. = boiled ham.

Action taken at the shop

The shop was revisited on 13 October and swabs were taken from various items of equipment, including the slicing machine. A number of unopened cans of chopped pork and minced pork and a partly used roll of minced pork which had lain on the same shelf as the suspected roll of chopped pork, were sent to the laboratory for bacteriological examination. *S. reading* was isolated from all parts of the slicing machine tested and from the counter shelf on which the suspected roll of chopped pork had lain. *S. reading* was also isolated from the external surface of the partly used roll of minced pork. Nothing was cultured from the contents of the unopened cans.

After sterilization with a hypochlorite solution, the equipment and surfaces in the shop were re-swabbed on two occasions, with negative results. At this visit on 13 October arrangements were made for bacteriological examination of all the shop assistants. On 20 October the grocer voluntarily closed the shop, because by then all five members of the staff were known to be excreting *S. reading*.

Analysis of cases

The total number of infected persons was 55, of whom 23 were males and 32 were females. Thirty-four had clinical symptoms of food poisoning and 21 were

asymptomatic excretors of *S. reading* (Table 1). Twenty-three had eaten chopped pork and 10 had eaten boiled ham purchased from the same shop. A further 14 had eaten neither chopped pork nor boiled ham (Table 2), and of these, ten could have been infected by close household contact with infected persons. The remaining four could not be linked with the outbreak. Ten persons were admitted to hospital, six as emergency admissions.

Treatment and follow-up

Ten persons were admitted to hospital for treatment, the remainder being treated at home by the family doctor. Neomycin sulphate was the antibiotic chosen initially in five of the hospital admissions and in all of the domiciliary cases. Three of the remaining hospital cases were given Ampicillin in a dosage of between 2 and 4 g. daily depending on the severity of symptoms. One hospital case was given no drug treatment. Six persons were given subsequent courses of treatment before a negative result was obtained.

Non-food handlers were allowed to return to their usual occupation after three consecutive negative stool specimens had been obtained. In the case of food handlers, six consecutive negative stool specimens were required although, owing to the pressure of business, the manager of the shop and one assistant were allowed to return to work after four consecutive negative stool specimens.

Conclusions

The shop had been established as the almost certain source of infection, and chopped pork as the vehicle, and consideration now had to be given to the question of how this food became contaminated. It was necessary at the earliest possible moment to prove that a carrier amongst the shop staff had not infected the meat after the can was opened.

Although all the shop assistants were found to be excreting *S. reading*, none had had symptoms of food poisoning before 10 October when the suspected meat was sold. One assistant, who developed symptoms shortly after this date, had been serving on the cold meat counter at the time the suspected meat had been on sale and had eaten some that morning. The only other shop assistant with gastro-enteritis became ill about a week after the meat had been sold.

Four 4 lb. tins of chopped pork were opened on 10 October, the first about 9 a.m. After opening, the contents of the tins were placed on a counter shelf in a window facing north-east. As a customer ordered it, the chopped pork was taken from the shelf, sliced on the slicing machine, and then replaced on the shelf which held other cold meats, including minced pork and boiled ham.

In this outbreak the vehicle of infection, i.e. either chopped pork or boiled ham, and its time of purchase can be fixed with reasonable accuracy and it is interesting to note that of the six emergency admissions to hospital, who were the persons most seriously affected, five had eaten chopped pork purchased on Saturday, 10 October about 10 a.m., approximately 1 hr. after the first tin of chopped pork had been opened.

Had the roll of chopped pork been contaminated by a carrier at the time of

opening it is unlikely that sufficient multiplication of organisms could have occurred in the short time available and this is made more unlikely by the fact that the roll was placed in a window facing north-east where conditions were not suitable for bacterial multiplication.

A study of the incubation periods reveals some interesting facts (Table 3). The mean incubation period in the case of those who had eaten chopped pork only was 31 hr. Two batches of chopped pork were involved, however. One batch was sold on Saturday, 10 October, and the other on Tuesday, 13 October, on the day before the sale of cold meats was forbidden and before the equipment had been sterilised.

Table 3. *Comparison of incubation periods of persons eating one or other or both cooked meats (first outbreak)*

Food eaten	No. of persons eating meats	No. of persons with incubation periods of			
		0–12 hr.	13–24 hr.	25–36 hr.	Over 36 hr.
Chopped pork	23	5	3	4	5
Boiled ham	10	0	1	1	3
Chopped pork and boiled ham	8	0	3	3	1
Total	41	5	7	8	9

If it is assumed that the original source of the outbreak was an infected tin of chopped pork opened and sold on Saturday, 10 October, around 10 a.m. (and four 4 lb. tins were opened on that day), then it might also be assumed that the contents of any further tins opened on that date would be contaminated from infected equipment and by manual handling. Persons eating chopped pork from the first infected tin bought on the morning of Saturday, 10 October, would probably have more severe symptoms and shorter incubation periods than persons affected by eating chopped pork bought on 13 October which had been contaminated in the shop from the slicing machine, the shelves, or by manual handling. This conclusion is supported by the duration of the incubation period, viz.:

Mean incubation period of persons eating chopped pork bought on 10 October 1964 was 19 hr.

Mean incubation period of persons eating chopped pork bought on 13 October 1964 was 69 hr.

The isolation of *S. reading* from the remains of the meat eaten by the family first affected and from a rectal swab taken from a member of the same family, and the absence of infection in the mother of the family who had not eaten the meat, would suggest that chopped pork was the source of the outbreak. When it is further considered that there was no clinical or bacteriological evidence to suggest the presence of a carrier among the shop staff before the opening of the original tin and that there was heavy and widespread contamination of the serving area, coupled with the evidence deduced from a study of the incubation periods, the conclusion is drawn that the source was a roll of chopped pork sold on 10 October between 10 and 11 a.m. Despite the original tin not being available for bacterio-

logical examination, it would appear likely that the meat was not contaminated by a salmonella carrier in the shop but was infected at some time during the canning process.

THE SECOND OUTBREAK

On 21 May 1965 the Public Health Department was notified by the University Bacteriology Department that a salmonella of group C (subsequently identified as *S. tennessee*) had been isolated from a man and his 3-month-old son. Inquiry showed that all five members of the family had had diarrhoea and vomiting the previous week-end and that a number of families in the area had had 'gastro-enteritis' about that time.

As investigations proceeded it became evident that minced pork and cold sliced pork, both bought from a supermarket in the area on 13 May, had been eaten by a number of persons affected and, accordingly, the shop was visited on the same evening as the first case was notified by the laboratory. Further investigation showed that a total of 39 persons from 17 families had a history suggestive of food poisoning.

The shop and staff

Five persons were employed in the shop and, on questioning, three admitted symptoms of diarrhoea and vomiting the previous week-end (15–16 May) while two denied having had any symptoms whatsoever. All denied ever having eaten cold meat at any time or having taken cold meat home with them. Stool specimens from all five yielded *S. tennessee* on culture.

Swabs were taken from a number of items of equipment in the shop and *S. tennessee* was isolated from the slicing machine, from the shelf on which the cold meats were put on display and from the refrigerator trays where the suspected cold meats were stored overnight.

Analysis of cases

The total number of persons involved was 39. Of these, 34 had symptoms and five were asymptomatic.

Of the 34 with symptoms, 31 yielded *S. tennessee* on stool culture and three did not. Stool cultures from the five asymptomatic cases were all positive for *S. tennessee*. Out of the group of 34 patients with symptoms, 17 had eaten canned minced pork and five had eaten cold pork sliced on the infected slicer in the shop. In the remaining 12 no history of having eaten pork could be obtained, although six of the 12 were members of the shop staff or their immediate family and could have been infected by contamination in the shop or at home.

Of the remainder, four were schoolchildren who had no direct connexion with the supermarket and had eaten neither minced nor sliced pork and two were infants aged 3 and 6 months, who were presumably infected by their parents who were positive cases. Stool cultures from all six children were positive for *S. tennessee*.

Incubation periods

The minimum incubation period was 10 hr. and the maximum 67 hr. Although the mean incubation period was 27 hr., the mean incubation period of those persons eating the suspected pork was 25 hr. and of those eating cold sliced pork 27 hr.

There were no hospital admissions, all cases being treated at home by family doctors.

DISCUSSION

In both outbreaks the suspected meats had been imported in cans. In the first outbreak the country of origin was Hungary and in the second Yugoslavia. Attempts to find out how the meats had been processed and under what conditions the meats had been canned met with little success.

The two outbreaks were also similar in that there was no evidence to suggest that a carrier amongst the shop staff was responsible for contaminating the meats.

The number of cases, 14 in the first outbreak and 12 in the second, who are presumed to have contracted their infection by close contact with others, may at first sight appear large but all of them were close family contacts of a known positive case and in the absence of any other source it would seem reasonable to presume that they contracted their infection in this way.

Table 4. *Numbers of patients excreting salmonellas for periods of 1 to 7 months after infection*

	Duration of excretion in months							
S. reading	1	1–<2	2–<3	3–<4	4–<5	5–<6	6–<7	7 and over
Symptomatic	13	10	4	0	0	1	0	1
Asymptomatic	12	4	1	0	0	2	0	2
Total	25	14	5	0	0	3	0	3
S. tennessee								
Symptomatic	16	2	2	1	3	0	0	0
Asymptomatic	3	1	0	1	0	0	0	0
Total	19	3	2	2	3	0	0	0

For five patients the duration of excretion is not known.

The four cases which could not be linked with the first outbreak were all children living in widely separated areas of the city and in the county of Midlothian. All, however, were positive for *S. reading* on stool culture.

In a number of patients infected with *S. reading* symptoms were severe and in some ways resembled paratyphoid fever. In one there was bacteraemia and in several there was generalized abdominal pain with tenderness in one or other iliac fossa. The similarity was also present in the length of time some cases continued to excrete the causative organism (see Table 4). Normally patients

who have made a clinical recovery from salmonella infection continue to excrete the organism in the faeces for varying periods. The average period of excretion for adults is about 6 weeks and the majority are clear of infection after 3 months.

In the first outbreak 25 persons (45·4 %) were clear of infection after 1 month, 44 persons (80 %) were clear of infection after 3 months and three persons (5·4 %) were still excreting the causative organism 6 months after contracting the infection and, of these, one was still excreting the organism 12 months after the initial infection.

It is interesting to note that in neither outbreak did a can show any external signs of contamination such as blowing, nor did the meat arouse suspicion, it being entirely free from obnoxious odour or discoloration. This is in keeping with evidence given to the Royal Commission on the Aberdeen Typhoid Outbreak by the Chief Veterinary Inspector for the City and Royal Burgh of Edinburgh (Report 1965, p. 38).

In both outbreaks, despite routine cleaning of equipment and surfaces, widespread contamination occurred in the shops. In the second outbreak this contamination extended to the table used by the staff at refreshment breaks, although it was situated some distance from the serving area. This table was presumably contaminated by the hands of the shop assistants, all of whom denied having taken cold meat to the table. It is easily seen how the spread of infection could be facilitated under such conditions. Improvements in the handling of cold meats in shop premises would minimize the risk of transmission to a certain extent, but the only certain course is to ensure that canned meats are rendered safe at source

SUMMARY

Two outbreaks of salmonella food poisoning are described. In one, involving 55 persons, the infecting organism was *Salmonella reading*, and in the other, involving 39 persons, *S. tennessee*. In both, epidemiological and bacteriological evidence pointed to imported cans of cooked meat as the source. The role of contaminated shop equipment in furthering the spread of infection is particularly emphasized.

Attention is drawn to the severity of the symptoms and prolonged excretion of the organism in a number of the patients infected with *S. reading*.

We would like to thank Prof. R. Cruickshank, Professor of Bacteriology, University of Edinburgh, and Dr J. L. Gilloran, Medical Officer of Health, City of Edinburgh, for their interest and encouragement in the preparation of this paper.

We wish also to acknowledge the help of the staff of the Infectious Disease Unit, City Hospital, Edinburgh, in providing details of patients who were admitted to hospital, and Miss J. Wallace for her valuable assistance.

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A comparative virological study of children in hospital with respiratory and diarrhoeal illnesses

By E. J. STOTT*, E. J. BELL, M. B. EADIE, C. A. C. ROSS and N. R. GRIST

Regional Virus Laboratory and University Department of Infectious Diseases, Ruchill Hospital, Glasgow, N.W.

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INTRODUCTION

Of the many viruses isolated from man in recent years, some show a clear-cut association with specific human disease, others with a range of illnesses, but many show little or no association with any deviation from health. The interpretation of the significance of virus infections diagnosed by laboratory tests can therefore be very difficult, particularly in the case of the increasing number of viruses detected in faecal and respiratory specimens by tissue-culture techniques.

One approach to this problem is the comparison of virus infections in groups of persons suffering from different clinical illnesses but otherwise comparable. This paper describes an investigation of this type. Virological studies of respiratory illness in children admitted to Ruchill Hospital, Glasgow (Ross, Stott, McMichael & Crowther, 1964) were extended to allow comparison with a group of children matched for age and time of admission to the same hospital but suffering from diarrhoeal illness. The results reported here are based on examinations of 113 children in each of the two groups admitted to hospital between October 1963 and April 1965.

MATERIALS AND METHODS

Selection of cases

Cases were selected on certain days of the week from those admitted to hospital within the previous 24 hr. Children suffering from respiratory illness were included only if they were under 10 years of age and could be matched for age (within a month if under 3 months old, within 6 months if aged 3–12 months, within 2½ years if over 1 year) and time of admission (within 1 week) with children with mainly diarrhoeal symptoms.

Collection of specimens

After selection, the children were immediately visited by one of us (M.B.E.) who collected from each a nose swab (a cotton-wool pledget packed into the nose for several minutes) and a throat swab. These were placed in separate bijou bottles each containing 2 ml. of transport medium consisting of Hanks's balanced salt solution (BSS) with 1 % bovine plasma albumen and 0·088 % sodium bicarbonate, 100 units penicillin/ml., 100 μg. streptomycin/ml. and 50 units nystatin/ml. After

* Present address: Common Cold Research Unit, Harvard Hospital, Salisbury, Wilts.

April 1964 nose and throat swabs were placed in the same bottle of transport medium and tested as one specimen. A request was left in the ward for faecal specimens to be collected as soon as possible; acute and convalescent blood sera were requested only from the respiratory cases.

Treatment of specimens

Nose and throat swabs were squeezed with forceps to express excess fluid into the transport medium which was centrifuged at 5000 r.p.m. Of the supernatant fluid 0·2 ml. was inoculated into each of two tubes of each of the following tissue cultures: 'Bristol' line of HeLa cells (BH), primary rhesus monkey kidney cells (RMK) and semi-continuous human embryo kidney fibroblasts (HEKF). The deposit was also inoculated into one tube of each of these cultures and, in alternate pairs of cases, additional antibiotics (100 units penicillin, 100 μg. streptomycin, and 50 units nystatin) were added to each tube containing the deposits. After April 1964 centrifugation was omitted and the additional antibiotics added to all inoculated tissue cultures. Nose and throat swabs were always inoculated within 2 hr. of collection.

Faeces were shaken with phosphate buffered saline (PBS) containing 250 units penicillin/ml. and 250 μg. streptomycin/ml. to form a 20 % suspension which was centrifuged at 3000 r.p.m. Of the supernatant fluid 0·2 ml. was inoculated into two tubes each of primary human amnion (AMN) and RMK. Faecal extracts were inoculated immediately unless tissue was unavailable in which case they were stored at -40° C.

Blood sera were stored at -20° C.

Tissue cultures

For nose and throat swabs

Primary RMK cells (Duncan, 1961) were grown in Eagle's minimum essential medium (MEM) supplemented with 5 % inactivated calf serum, 0·044 % sodium bicarbonate, 100 units/ml. penicillin, and 100 μg./ml. streptomycin. Before inoculation cultures were washed three times in PBS and changed to Parker's 199 medium containing 0·18 % sodium bicarbonate, similar antibiotics but no serum. After inoculation these cultures were rolled at 33° C. and observed for cytopathic effects (CPE) three times weekly and tested for haemadsorption with human group 'O' erythrocytes once weekly for 3 weeks.

The HEKF cultures used in this study were a semi-continuous cell strain derived in this laboratory from a 10-week-old male foetus by methods similar to those described by Hayflick & Moorhead (1961). The cells were grown in Eagle's MEM containing 5 % inactivated precolostral calf serum or 10 % unheated calf serum, 0·088 % sodium bicarbonate, 100 units penicillin/ml., 100 μg. streptomycin/ml. For virus isolation cultures were maintained in Eagle's MEM with 1 % inactivated precolostral serum, 0·044 % sodium bicarbonate, 5 % tryptose phosphate broth and the usual antibiotics. After inoculation these cultures were rolled at 33° C., examined for CPE and the medium changed three times per week for 2 weeks.

The BH cells were obtained from the Common Cold Research Unit, Salisbury, and were grown in Hanks's BSS with 0·5% lactalbumen hydrolysate, 5% inactivated rabbit serum, 0·088% sodium bicarbonate and the usual antibiotics. Before inoculation the cultures were changed to a similar medium containing 2% inactivated fowl or rabbit serum instead of 5% rabbit serum and 0·13% sodium bicarbonate. After inoculation these cultures were held stationary at 36° C. and examined and the medium changed three to four times weekly for 3 weeks, when they were stained with neutral red (1/80,000) and discarded if no syncytia were apparent.

For faecal extracts

Primary RMK cells were grown in Hanks's BSS with 0·25% lactalbumen hydrolysate, 10% calf serum, 10% human serum, 0·044% sodium bicarbonate and the usual antibiotics. For the growth of AMN cells a similar medium was used, containing 20% human serum but no calf serum (Duncan & Bell, 1961). Before attempted virus isolation both types of cultures were washed once in PBS and changed to Eagle's MEM containing 1% calf serum, 0·176% sodium bicarbonate and antibiotics. After inoculation the tubes were held in a stationary sloped position at 36° C. and observed daily for CPE for 2 weeks.

Serological methods

Complement-fixation tests

All paired acute and convalescent sera were tested for complement-fixing (CF) antibodies against respiratory syncytial (RS) virus, the adenovirus group and parainfluenza type 1 virus by the methods described by Grist, Ross, Bell & Stott (1966); some paired sera were also tested with herpes simplex antigen. The antibody titre of each serum was taken as the reciprocal of the highest dilution of serum showing complete or almost complete fixation of complement. A titre was considered as rising if there was a fourfold or greater increase in titre or an increase from < 8 to 16 between the 'acute' and 'convalescent' serum.

Neutralization tests

Paired sera of children from whom RS virus was isolated were tested for neutralizing antibodies by the methods of Grist *et al.* (1966). The Randall strain of RS virus was diluted in PBS containing 25% unheated rabbit serum unless otherwise stated. The serum-virus mixtures were inoculated into BH cultures; after 3 days these were changed into fresh maintenance medium containing 1/80,000 neutral red, and read the following day when the virus titration indicated that 32–320 TCD 50 was present.

Paired sera from children from whom rhinoviruses were isolated were titrated for neutralizing antibodies against the homologous rhinovirus. The tests were performed in HEKF and read the day after the virus titration indicated that 10–32 TCD 50 had been used.

The antibody titre was taken as the reciprocal of the highest dilution in the serum-virus mixture which completely neutralized the virus.

Identification of viruses

RS virus, parainfluenza viruses 1–3, herpes simplex virus and adenoviruses 1–7 were initially grouped by their characteristic CPE and were finally identified by neutralization tests with type specific antisera.

Enterovirus strains were identified by neutralization tests using antisera to poliovirus types 1–3, coxsackievirus types A 9 and B 1–6, and echovirus types 1–25. The 'pooled-serum' technique of Lim & Benyesh-Melnick (1960) was used for the preliminary identification of the strains. Final confirmation of their identity was made with type specific antisera.

Viruses which produced enterovirus-like CPE most rapidly in RMK or HEKF when the cultures were rolled at 33° C. in a low bicarbonate medium were provisionally classified as rhinoviruses: M types if they produced CPE in both RMK and HEKF, H types if they grew only in HEKF. Such viruses were then tested for chloroform stability (Feldman & Wang, 1961) and acid lability (Tyrrell & Chanock, 1963). Viruses found to be chloroform-stable and acid-labile were considered to be rhinoviruses.

Identification of rhinoviruses was attempted by neutralization tests using antisera against the following 27 types: echovirus 28, Salisbury strains B 632, HGP, FEB, Thomson, Norman, 16/60 (Taylor-Robinson & Tyrrell, 1962) NIH strains 353, 1059, 1734, 11757, 363, 1200, 33342 (Johnson & Rosen, 1963 and Webb, Johnson & Mufson, 1964) Chicago strains 106-F, 140-F, 179-E, 127-1, 137-3, 164A (Connelly & Hamre, 1964 and Hamre, Connelly & Procknow, 1964) West Point strains 1, 68, 181, 204, 5986, MRH (Ketler, Hamparian & Hilleman, 1962) Baylor type 3 (Phillips, Melnick & Grim, 1965). The antisera were diluted to contain approximately 20 antibody units per 0·1 ml.

RESULTS

Population studied

The 113 children in the respiratory group represented 13 % of the children under 10 years of age admitted to this hospital with respiratory illnesses during the 19-month period. They comprised 64 males and 49 females aged from 3 weeks to 6 years with an average of 17·8 months. This was similar to the diarrhoea group which comprised 61 males and 52 females aged from 1 week to 4½ years with an average of 17·4 months (Table 1).

Three of the children in the respiratory group also had mild enteric symptoms on admission. Eight of the children in the diarrhoea group had some upper respiratory symptoms.

Virus isolation

The viruses isolated from the throat and nose are shown in Table 2. 64 viruses were isolated from 59 (52 %) of 113 children in the respiratory group and 31 viruses from 29 (26 %) of 113 children in the diarrhoea group. Faeces were available from 106 of the respiratory group and 110 of the diarrhoea group (Table 3) and enteroviruses were isolated from similar numbers in both groups (16 % and 19 %

respectively). The same virus was isolated from the throat and nose swabs and faeces in two respiratory cases (coxsackievirus B4 and echovirus 9) and in three diarrhoeal cases (adenovirus 3, echovirus 8 and an untyped enterovirus).

Table 1. *Population studied*

Group	Total cases	Age in years		
		under 1	1–2	2–6
Respiratory: Male	64	27	15	22
Female	49	23	11	15
Diarrhoeal: Male	61	24	16	21
Female	52	22	14	16

Table 2. *Viruses isolated from throat and nose swabs*

Type of case	No. of cases	RS	PF		HS	RH		AD					Polio	Coxsackie		Echo		UT	Total
			1	3		M	H	1	2	3	5	7	2	B2	B4	8	9		
Respiratory	113	26	2	7	12	1	9	2	2	1	0	0	0	0	1	0	1	0	64*
Diarrhoeal	113	1	0	2	4	1	9	2	1	1	3	1	1	2	0	1	1	1	31†

* Dual isolation in five cases: RS + herpes, Ad 1 + herpes, Rh M + herpes, Pf 3 + Ad 2, RS + Ad 1.
† Dual isolation in two cases: Rh H + Ad 5, Pf 3 + RS.
PF = parainfluenza, RH = rhinovirus, HS = herpes simplex, AD = adenovirus, UT = untyped enterovirus.

Table 3. *Viruses isolated from faeces*

Type of case	No. of cases	AD		Polio	Coxsackie			Echo								UT	Total
		1	3	3	A9	B3	B4	3	4	7	8	9	11	12	14		
Respiratory	106*	1	0	0	1	1	2	0	2	2	2	1	0	2	1	2	17
Diarrhoeal	110*	0	1	1	1	0	0	5	1	1	3	0	3	0	2	3	21

* Faeces unavailable from seven children in respiratory group and three in diarrhoea group.

Table 4. *Viruses isolated from throat and nose swabs of* 113 *children with respiratory disease according to day of illness*

	Day of illness			
	1–3	4–7	>7	Unknown
Cases tested ...	60	38	10	5
Viruses isolated ...	30 (50 %)	27 (71 %)	4	3
RS virus	12	11	2	1
Parainfluenza	4	3	1	1
Rhinoviruses	4	5	1	0
Herpes simplex	7	4	0	1
Adenoviruses	2	3	0	0
Enteroviruses	1	1	0	0

Analysis of the virus isolations from the throat and nose of children with respiratory disease in relation to the number of days between the onset of symptoms and the collection of swabs (Table 4) shows that viruses were isolated more

often from children who had been ill from 4 to 7 days than from those who had only been ill 3 days or less. During the first 7 months of the study different viruses were never isolated from the throat and nose swabs of the same patient. Virus was isolated from the nose alone in three cases, from the throat alone in 16 cases, and from both the throat and nose in 23 cases. We also found between October 1963 and April 1964 that the addition of extra antibiotics (100 units penicillin, 100 μg. streptomycin and 50 units nystatin) to each tissue culture tube inoculated with throat and nose swabs suppressed bacterial and fungal contamination as effectively as centrifugation. The examination of the supernatant and deposit after centrifugation yielded similar results except for five specimens where the supernatant was negative but the deposit yielded RS virus.

Serological tests by complement-fixation

Paired sera were received from 69 of the 113 respiratory cases. A rise in CF antibodies to RS virus was detected in 11 (16%) from eight of which RS virus was isolated; high ($\geqslant 128$) but not rising titres were found in seven of the remaining cases but only one of these yielded RS virus. Rising titres to the adenovirus group were found in four cases (two positive for adenovirus by isolation) and to parainfluenza type 1 in three cases (one yielded parainfluenza type 3); high ($\geqslant 64$) titres to adenovirus group were found in six (one positive for adenovirus by isolation) and to parainfluenza type one in two cases. Thus, infections diagnosed only serologically comprise three RS virus, two adenovirus and two parainfluenza virus.

Table 5. *Virus infections of* 226 *children according to illness*

Illness	Cases	Infections diagnosed	RS	PF	RH	HS	AD	ENT
Respiratory group								
Pneumonia and bronchopneumonia	47	41 (5)	13	6	4	6	6	6
Bronchiolitis and bronchitis	51	34 (3)*	15	3	3	4	1	8
Croup	3	2 (1)	0	0	0	1	0	1
U.R.T.I.	12	9 (1)	1	2	3	1	1	1
Diarrhoea group								
Bacterial diarrhoea	55	24 (5)	1	1	6	2	3	11
Non-specific diarrhoea	58	25	0	1	4	2	5	13
Total	226	135 (15)*	30	13	20	16	16	40

Figures in brackets are cases with dual infections.
* Includes one case with triple infection.
ENT = enterovirus, other abbreviations as in Table 2.

Type of illness

The relationship between various disease syndromes and total virus infections is shown in Table 5. RS virus infections were mainly found in cases of bronchopneumonia and bronchitis but illnesses associated with parainfluenza viruses were more varied. Rhinoviruses were associated with lower respiratory tract disease in seven cases but were isolated with the same frequency from both respiratory

and diarrhoeal illnesses. Adenovirus and enterovirus infections were found almost as often in children with respiratory illnesses as in those with diarrhoea. Similar numbers and types of virus infections were found in cases with either bacterial or non-bacterial diarrhoea.

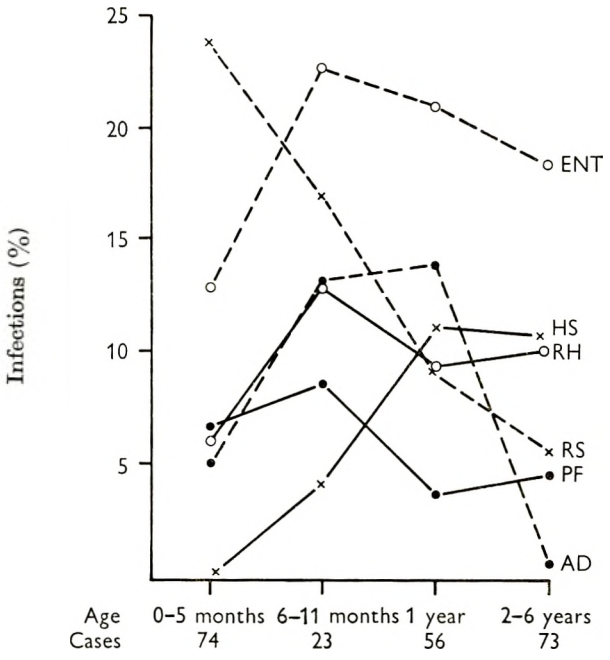

Fig. 1. Distribution of percentage infections with various viruses according to age.

Age of patients

The age distribution of the 135 virus infections in shown in Fig. 1. Most of the RS virus and parainfluenza virus infections were found in children under 1 year of age. Herpes simplex infections were most common after the first year of life and adenovirus infections between 6 months and 2 years. Infections with enteroviruses and rhinoviruses were fairly evenly distributed throughout the age range studied.

Monthly distribution

The RS virus infections, with one exception, occurred during two separate 5-month periods which correlated well with the peak months for admissions of children with respiratory disease (Fig. 2). Infections with all the other viruses studied occurred sporadically throughout the year.

Respiratory syncytial virus infections

RS virus was isolated from 26 respiratory cases but from only one diarrhoeal case (which was also infected with parainfluenza type 3 virus and developed a severe cough with upper and lower respiratory tract signs 3 days later), giving a highly significant difference ($\chi^2 = 25{\cdot}8$, $P < 0{\cdot}001$); this indicates that RS virus is closely associated with respiratory illness (Table 2). In addition, rising CF antibody

titres for RS virus were found in three cases from which no virus was isolated. If these are included as RS virus infections 29 (26 %) of the 113 respiratory illnesses were associated with RS virus and 25 (86 %) of them were under two years of age.

Two or more blood sera were received from 14 of the cases from which RS virus was isolated; the titres of CF and neutralizing antibodies are shown in Table 6. The CF test detected a rising titre to RS virus in eight cases and high titres

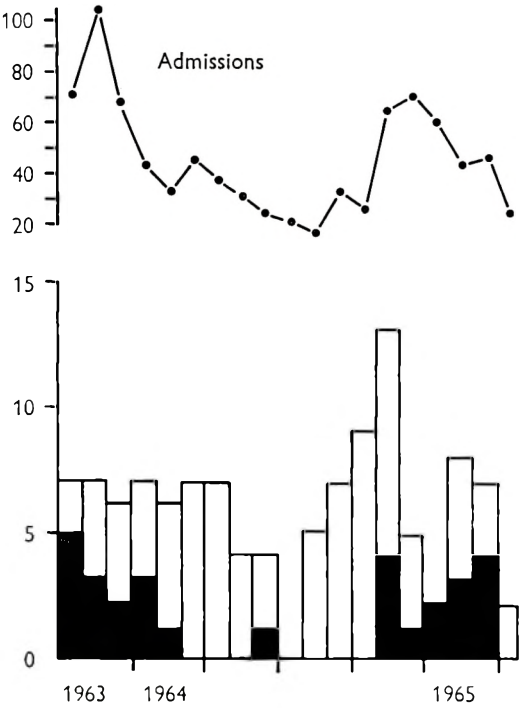

Fig. 2. Monthly distribution of children admitted to hospital with respiratory illness, cases studied and RS virus infections □, Cases studied; ■, RS virus infections.

(128;256) in one case. These children were at least 4 months old, in contrast to the five cases under 4 months in which no CF antibodies were detected. Of eight patients with rising CF titres, seven were also tested for neutralizing antibodies; they all showed titre rises provided unheated rabbit serum was present in the serum-virus mixtures. The absence of unheated serum or its inactivation at 56° C. for 30 min. reduced neutralizing titres two- to eightfold and prevented the detection of rising titres in four of six cases tested. Neutralizing activity without change in titre was obtained with sera from two 2-month-old babies; no antibodies were found by the CF test. The titres of neutralizing antibodies to two strains of RS virus, one isolated during each of the outbreaks, were similar to those obtained with the Randall strain (even in the two cases from whom these strains were isolated).

Parainfluezna virus infections

Parainfluenza viruses were isolated from nine children in the respiratory group (two type 1 and seven type 3) and two (type 3) in the diarrhoeal group (Table 2)

showing a significant difference between the two groups ($\chi^2 = 4{\cdot}68$, $P < 0{\cdot}05$). Two additional children in the respiratory group also had a titre rise with parainfluenza type 1 antigen in the CF test, but as there are frequent cross-reactions between the parainfluenza viruses and mumps virus, these antibody responses cannot be considered specific.

Table 6. *Serological results in* 14 *children from whom RS virus was isolated*

			Neutralization titres			
			Randall strain		Epidemic strain*	
Age (months)	Day of illness	CF titres	With rabbit serum	Without rabbit serum	With rabbit serum	Without rabbit serum
1	1	ND†	‡			
	9	<8	—	—	—	—
2	5	ND	32			
	38	<8	32	—	—	—
2	6	ND	32	8	32	<8
	16	<8	32	8	32	<8
3	4	ND				
	11	<8	—	—	—	—
3·5	3	ND				
	18	<8	—	—	—	—
4	3	<8				
	25	16	—	—	—	—
4	3	8	16	8	**16**	**8**
	11	16	64	8	**64**	**16**
	30	128	128	—	**128**	—
5	10	128	<16	<16	<8	<8
	41	256	64	16	128	<16
5	5	<8	<8	—	—	—
	14	16	64			
7	5	<8	8	<8	<8	<8
	12	512	⩾256	128	⩾256	64
7	1	<8	<8	<8	<8	<8
	12	16	32	<16	32	<16
11	3	<8	<8			
	10	128	⩾256	—	—	—
11	4	<8	<8	<8	**<8**	**<8**
	16	16	64	8	**64**	**<8**
13	6	<8	<8	<8	<8	<8
	13	128	64	16	64	16

Figures in bold type are titres of sera from patients from whom strains 121–64 and 143–65 were isolated.

* For 1963–4 winter epidemic strain was 121–64 and for 1964–5 winter, strain 143–65.

† ND = not done.

‡ = insufficient.

Rhinovirus infections

Rhinoviruses were isolated from 10 cases of respiratory illness and 10 cases of diarrhoeal illness (Table 2). Of the latter, five had no record of respiratory illness before or after entry into hospital, one had a cold 6 days before entering hospital but no symptoms on admission, three had a cough on admission and one developed a cold the day after entering hospital. None of the rhinoviruses isolated from diarrhoeal illnesses was neutralized by the 27 antisera used.

Table 7. *Details of* 10 *respiratory cases with rhinovirus infection*

Age (months)	Sex	Illness	Type and serotype	Day of illness	Neutralization titre to rhinovirus
1·5	M	U.R.I.	H, UK*	—	—
3	M	Pneumonia	H, UK	—	—
4	F	Bronchitis	H, UK	10	16
				24	128
7	M	U.R.I.	H, 181	—	—
11†	F	Bronchitis	M, B632	9	<8
				18	128
12	M	Bronchitis	H, UK	8	
				17	—
21	M	Bronchopneumonia	H, UK	2	<2
				10	2
24	M	Pneumonia	H, UK	—	—
34‡	F	U.R.I.	H, UK	2	<8
				9	<8
36	M	Pneumonia	H, 5986	1	<8
				23	32

* UK = serotype unknown.

† Herpes simplex virus also isolated from throat and nose and echovirus 7 from faeces of this case.

‡ CF antibody titres to RS virus rose from < 16 to 64.

Details of the 10 respiratory cases yielding rhinoviruses are shown in Table 7. In seven the lower respiratory tract was involved. Apart from one case of upper respiratory infection which showed a greater than fourfold rise in CF antibodies against RS virus, no evidence of simultaneous infection with RS virus, adenovirus or parainfluenza type 1 virus was found in the six cases with paired sera. Three rhinoviruses were neutralized by specific antisera suggesting that they were serologically identical with strains B632, 181 and 5986 respectively. A fourfold or greater titre rise in antibody to the patient's own virus was detected in three cases; in two cases where the second serum was collected less than 2 weeks after the onset of illness, a low level of antibody was found in one and no antibody in the other.

Herpes simplex virus infections

Herpes simplex virus was isolated significantly more often from the respiratory group than from the diarrhoeal group (Table 2). However, paired sera from five of the infected children showed stationary levels of CF antibody against herpes virus.

Adenovirus infections

Adenovirus types 1, 2 or 3 were isolated from the throats of five children and faeces of another in the respiratory group and the throats of four in the diarrhoea group, one of whom had the same virus (type 3) in the faeces (Tables 2 and 3). Three children in the diarrhoeal group had adenovirus type 5 in the throat and one had type 7. There were an additional two children in the respiratory group who had fourfold or greater CF titre rises against adenovirus. Thus, there were 8 adenovirus infections in each of the two groups (Table 5). Paired sera collected from five of the six children in the respiratory group from whom virus was isolated showed a fourfold or greater rise in CF titres for adenovirus in only two.

Table 8. *Multiple infections*

Illness	Throat/nose swab	Faeces	Serological
Tonsillitis	RH H	—	RS
Croup	HS	Echo 4	—
Bronchitis	—	—	AD + PF
	HS + RS	—	—
	HS + RH M	Echo 7	—
Bronchopneumonia	HS + AD 1	—	—
	AD 1 + RS	—	—
	AD 2 + PF 3	—	—
	PF 3	AD 1	—
	RS	Cox. B 4	—
Dysentery	AD 1	UT	—
	AD 5 + RH H	—	—
	RH H	Cox. A 9	—
	RH H	UT	—
Gastroenteritis	RS + PF 3	—	—

Enterovirus infections

There were 16 enterovirus infections in the respiratory group and 24 in the diarrhoeal group (Table 5). In the respiratory group coxsackieviruses were isolated from four cases and echoviruses or untyped viruses from 12; both respiratory and faecal specimens of two cases yielded the same virus (coxsackievirus B4 and echovirus 9 respectively). The 24 enterovirus positive cases in the diarrhoeal group comprised two polioviruses, three coxsackieviruses, 16 echoviruses and three untyped enteroviruses. Throat and nose and faecal specimens both contained virus in one echovirus 8 case and one case with an untyped virus (Tables 2 and 3).

Multiple infections

Multiple infections (Table 8) were found in 10 of the children with respiratory illness and five of those with diarrhoeal illness. Herpes simplex was one of the infecting agents in four of the 10 respiratory cases and adenovirus was found with another viral infection in five respiratory cases and two diarrhoeal cases.

DISCUSSION

The strict criteria for matching children with respiratory illness with those suffering from diarrhoeal illness for age and time of entry into hospital limited the number of cases available for study. Nevertheless, immediate inoculation without prior freezing of throat and nose specimens into three types of tissue cultures enabled us to isolate a large number of viruses. Collection of these specimens within 24 hr. of admission allowed little opportunity for hospital cross-infection which is thought to occur with RS virus (Chanock *et al.* 1961). Thus, despite the small numbers studied certain conclusions about the epidemiology of respiratory viruses can be made.

Our findings that of the children with respiratory disease 26 % had evidence of infection with RS virus and 10 % with parainfluenza viruses and that these viruses were isolated more often from children with respiratory disease than from those with diarrhoeal disease agree broadly with the larger studies of others (Holzel *et al.* 1965; Clarke *et al.* 1964; Chanock & Parrott, 1965).

The isolation of rhinoviruses from 7 % of children with lower respiratory illness is a slightly higher rate than the 3·1 % found by Bloom, Forsyth, Johnson & Chanock (1963) and the 4·2 % found by Portnoy, Eckert & Salvatore (1965), in similar groups. This may be partly due to the use of HEKF which revealed 30 % more rhinoviruses when used in parallel with WI 38 cells (unpublished data). The rhinovirus isolation rate of 8·8 % in the diarrhoeal group is also higher than that found in the control group of other studies, but this may be partly due to the inclusion in our diarrhoeal group of some cases with mild respiratory symptoms. Hamparian, Leagus, Hilleman & Stokes (1964) reported the isolation of rhinoviruses from 14 children with lower respiratory disease but did not show a causal relationship although an earlier report from this group (Reilly *et al.* 1962) stated that cases selected for rhinovirus study were among those whose paired sera failed to give evidence of infection with influenza, parainfluenza, reovirus (group), adenovirus (group), and RS virus. Portnoy *et al.* (1965) found serological evidence of simultaneous infection with RS virus or parainfluenza type 3 virus or both in five of 13 (38 %) children with lower respiratory disease associated with rhinovirus infection. Serological tests on six of our cases with rhinovirus infection revealed only one with evidence of infection with another (RS) virus. Thus, we did not obtain convincing evidence that rhinoviruses caused lower respiratory disease in this group of children although the ability of these viruses to infect the lower respiratory tract was shown in adult volunteers by Cate *et al.* (1965) and also suggested by our own study of adults with chronic bronchitis (Eadie, Stott & Grist, 1966).

Although herpes simplex virus was isolated significantly more often from the children with respiratory illness than from those with diarrhoeal illness, the stationary antibody levels in the five respiratory cases tested suggest that these were not primary infections with herpes virus, but reactivated latent infections secondary to, not a cause of, the respiratory illness.

Neither adenovirus nor enterovirus infections showed any significant difference between the two groups. This may be due either to low pathogenicity of these viruses or to their ability to cause both respiratory and diarrhoeal symptoms. The infection rates (Table 5) for enteroviruses of 21 % for children with diarrhoeal illness and 14 % for children with respiratory illness are closely similar to those found by Sommerville (1958) in a previous study in this hospital and do not suggest that enteroviruses were significant causes of diarrhoeal illness during our study.

In a previous study (Ross *et al.* 1964) development of CF antibodies to RS virus was found to be slow in some children, the highest titre not being attained until between the fourth and sixth week of illness. In the present study many of the convalescent sera were collected in the second to third week of illness before the children left hospital; this may explain our failure to detect a rise in CF antibody to RS virus in five children from whom RS virus was isolated. However, as all these five were under 4 months of age this might support the finding of previous workers (Beem *et al.* 1960; Chanock *et al.* 1961; Gardner, Elderkin & Wall, 1964) that a CF antibody response to RS virus infection is rarely detected during the first few months of life. In our earlier study differences in neutralizing antibody titres had been detected by the use of a strain of RS virus isolated locally but we could not repeat this finding with strains isolated during the present study. This discrepancy could be explained by differences of 'avidity' or antigenic variation between the three local strains used; work is in progress to elucidate this point for although antigenic differences have been found between RS virus strains these have not been detectable in neutralization tests with human sera (Coates, Kendrick & Chanock, 1963; Doggett & Taylor-Robinson, 1965). The value of unheated rabbit serum in the measurement of neutralizing antibodies against rubella virus has been reported by several workers (Neva & Weller, 1964; Plotkin, 1964; Parkman, Mundon, McCown & Buescher, 1964), but our finding that RS neutralizing antibody titres could be increased two- to eightfold by the addition of unheated serum does not appear to be widely known.

SUMMARY

Between October 1963 and April 1965, 113 children with respiratory disease and 113 children with diarrhoeal disease were matched for age and time of entry into hospital and studied by virus isolation and serological techniques.

Infections with respiratory syncytial (RS) virus, parainfluenza virus and herpes simplex virus respectively were found in 29, 11 and 12 children in the respiratory illness group but in only 1, 2 and 4 children in the diarrhoeal group. Rhinoviruses were isolated from 10 children in each group and in seven cases were associated with lower respiratory disease. Adenovirus infections were found in nine children

with respiratory disease and eight with diarrhoea. Of the 40 enteroviruses isolated 16 were associated with respiratory disease and 24 with diarrhoea.

A poor or delayed serological response in children under 4 months with RS virus infection was observed. Addition of unheated rabbit serum increased the sensitivity of the neutralization test with RS virus.

These findings indicate that respiratory syncytial and parainfluenza virus infections were clearly associated with respiratory illness but the pathogenic role of the other viruses was not clear.

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Respiratory illness and viral infection in an Edinburgh nursery

By C. J. D. AITKEN,* MARGARET A. J. MOFFAT
AND J. A. W. SUTHERLAND
Wellcome Laboratory, City Hospital, Edinburgh

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In recent years many outbreaks of respiratory syncytial virus infection have been reported and the associated clinical syndromes are now well documented (Chanock *et al.* 1962; Hilleman *et al.* 1962; Holzel *et al.* 1965). Similarly many accounts of respiratory illness resulting from infection with adenovirus (Bell *et al.* 1955; Chany *et al.* 1958; Hilleman *et al.* 1962), parainfluenza virus (Hilleman *et al.* 1962; Parrott, Vargosko, Kim & Chanock, 1963) and influenza virus (Balducci, Zaiman & Tyrrell, 1956; Holland, Tanner, Pereira & Taylor, 1960) have been published. However, few investigations on these or other respiratory virus infections have been carried out on static populations of normal subjects who were undergoing continuous clinical observation. In this country, Sutton (1962) reported outbreaks of influenza A, parainfluenza 3 and adenovirus infections occurring over a period of 8 months in a nursery containing forty-six children. In the U.S.A., Bell, Rowe & Rosen (1962) described the illnesses occurring in 587 children of the Washington Children's Village over a period of 3 years and described outbreaks of infection with adenoviruses, myxoviruses and coxsackieviruses. Kapikian *et al.* (1961) extensively documented an outbreak of respiratory syncytial virus infection in the Washington Children's Village among ninety children who were under continuous clinical observation.

The present survey was carried out in a community of resident nursery children in Edinburgh during the winter 1963–64 to investigate the prevalence and type of virus infection, as proved by serological means, and to relate this to the respiratory illnesses occurring at the time.

MATERIALS AND METHODS

The population at risk

Forty-nine children were resident in the nursery during the 6 months of the study. Forty-one of these children were included in the final analysis. The remaining eight were excluded either because they were admitted for less than 1 week and during that time remained isolated from the other children in the admission ward, or because they were admitted for less than 1 month and an insufficient number of specimens was obtained from them. The remaining 41 remained in the nursery for at least 11 weeks and 13 were resident in the nursery during the whole 26 weeks of the study. The average duration of the study per child was 18·4 weeks and the total period of observation was 764 child weeks. There were 14 admissions and

* Present address: Leith Hospital, Edinburgh.

14 discharges during the survey. The sex ratio of children was 25 males to 16 females but their total duration in the nursery was more diverse, i.e. 487 male child weeks to 277 female child weeks.

In the nursery the children were organized into three groups:

(1) *Babies.* Eight babies were included in the study, their ages ranging from 3 to 28 weeks with a mean age of 16·3 weeks. The babies were cared for in one room and had little or no contact with the other children.

(2) *Infants.* Seventeen infants were included in the study, their ages ranging from 7 to 18 months apart from one child aged 28 months who was mentally retarded. They had a mean age of 14·3 months. These children remained separate from the other groups for eating and sleeping but a few of the older children mixed with the toddler group during play.

(3) *Toddlers.* Sixteen toddlers were included. Their ages ranged from 20 to 44 months with a mean age of 32·6 months.

The staff circulated freely among all groups.

Methods of assessment of illness

The nursery was visited twice a week by the same observer (C.A.) and a report taken from one of the three senior members of the staff concerning any ill child. These children were examined. Similarly the progress of any ill child was followed by repeated clinical examinations and reports by the same member of the staff.

The following categories of respiratory illness were recognized:

(1) *Upper respiratory tract infection* (U.R.T.I.). A respiratory illness confined to the nasopharynx showing one or more of the following signs: mucoid nasal discharge, purulent nasal discharge, pharyngitis, cervical lymphadenopathy, middle ear infection, or systemic upset.

(2) *Bronchitis.* A respiratory illness running a benign course, characterized by cough usually associated with signs of lower respiratory tract infection (crepitations, rhonchi, audible wheeze or mild respiratory difficulty) and with systemic upset with or without pyrexia and signs of upper respiratory tract infection.

(3) *Bronchiolitis.* A respiratory illness occurring usually in babies, running a severe course and associated with severe respiratory distress. There were no cases of bronchiolitis during the survey.

(4) *Pneumonia.* A severe lower respiratory tract illness, requiring hospital admission and having at least some radiological evidence of pulmonary consolidation.

(5) *Laryngo-tracheo-bronchitis* (L.T.B.). A respiratory illness, clinically confined to the larynx, trachea and main bronchi characterized by persistent cough, inspiratory stridor, dysponea and pyrexia.

An episode of illness had to be present for 24 hr. or more to be included in the results. The last day before the return of the child to his normal health was considered to be the last day of illness. A period of at least 48 hr. free from signs and symptoms of respiratory disease was required to separate any two periods of illness. Some of the children showed a persistent nasal discharge during the whole survey. These children were only considered to have a new respiratory illness if

there was an increase or change in their symptoms. The child's age was recorded on the first day of the illness, to the nearest week in the case of babies and to the nearest month in the case of the infants and toddlers.

Collection of specimens

During the first fortnight of the survey pharyngeal swabs and blood specimens were obtained from each child. Thereafter routine pharyngeal swabs were taken every 4 weeks and routine blood specimens every 8 weeks. Pharyngeal swabs and blood specimens were taken from each child at the beginning of an illness (acute specimens) and again 2 weeks later (convalescent specimens). To minimize the trauma to the children acute and convalescent specimens of blood were not taken if routine blood collection had occurred (in the case of acute specimens) or was to occur (in the case of the convalescent specimens) within 2 weeks. This meant a sacrifice in the accuracy of timing of viral infections but it was felt there would be no loss in the number of antibody rises obtained.

Virological methods

Throat swabs were immediately immersed in 3 ml. of transport medium which consisted of medium 199 containing bovine serum albumin (1%), sodium bicarbonate (5% of a 4·4% solution), penicillin (250 units/ml.) and streptomycin (250 μg./ml.). These specimens were transported to the laboratory in a cooled thermos flask with the minimum of delay and were kept at 4° C. until inoculation was carried out; in no case was more than 3 hr. allowed to elapse between collection and inoculation of the specimen. The specimens were inoculated into two cultures of HEp-2 cells maintained in 199 and 2% fowl serum and into two cultures of monkey kidney cells maintained in Earle's basic salt solution supplemented with 7·5% liver digest ultrafiltrate (Smith, 1961). The remainder of the specimen was then frozen quickly in alcohol at −70° C. and stored until human embryo lung cells were available. A strain of human embryo lung cells was maintained on Eagle's medium with 2% calf serum and low bicarbonate concentration, i.e. 1% of a 4·4% solution. All cultures were rolled at 34° C. and were examined every 2 days for 10 days; a further blind passage was then carried out on these cultures after freezing and thawing at −70° C. Haemadsorption tests using 0·4% human group 'O' erythrocytes were carried out on all monkey kidney cultures after 5 days on the second passage and again before discarding.

Sera were inactivated at 56° C. for 30 min. and stored at −20° C. They were tested for complement-fixing antibodies according to the technique described by Bradstreet & Taylor (1962) using the following antigens: influenza A, B and C, Sendai, parainfluenza 1, 2 and 3, adenovirus and respiratory syncytial virus. All complement-fixing reagents except the respiratory syncytial antigen were obtained from the Standards Laboratory for Serological Reagents, Central Public Health Laboratory, Colindale. The respiratory syncytial antigen was prepared in monkey kidney cells grown in serum-free medium. The seed virus was the Randall strain of virus previously passaged in HEp-2 cells. Sera were also tested for the presence of cold agglutinins to human group 'O' erythrocytes.

Neutralization tests were carried out on patient's serum against any virus isolated from that patient. The virus, diluted to contain 100 TCD50/0·1 ml., was mixed with 0·25 ml. amounts of twofold dilutions of serum. The mixtures were allowed to stand for 1 hr. at room temperature and then 0·2 ml. amounts were inoculated into each of two tubes of the appropriate tissue culture. The neutralizing titre was taken as the highest dilution showing inhibition of the cytopathic effect. Identification of virus strains was carried out by neutralization tests using the following antisera: poliovirus 1, 2, 3; coxsackie B 1–6; A 7, A 9; echo 1–10, 12, 14–16, 18–20, 22, 25, 26; adenovirus 1–11, 14–16, herpes simplex. Parainfluenza viruses were identified by haemadsorption inhibition tests.

RESULTS

Those children who showed a fourfold or greater rise in complement-fixing antibody were considered to have had a virus infection, whether the virus was isolated or not.

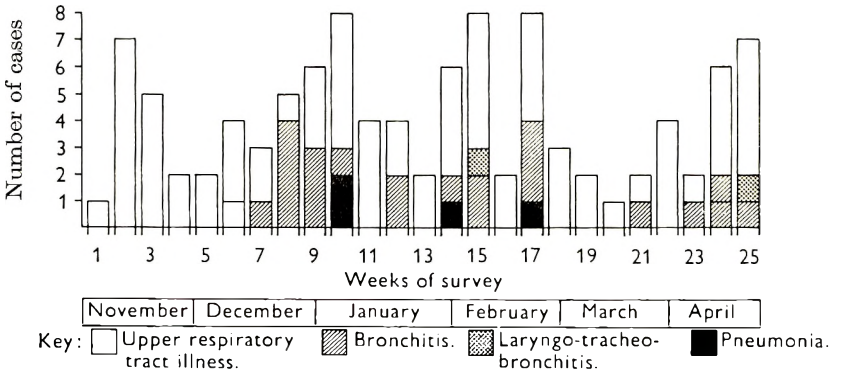

Fig. 1. The incidence of fresh cases of respiratory illness throughout the survey (November 1963 to April 1964).

During the 6 months of the survey there were 105 episodes of respiratory illness (2·6 per child) and of these 44 (42%) were associated with one or more virus infections. Evidence of virus infection was obtained on 67 occasions, 20 children showing no respiratory illness at the time and 3 having evidence of infection with two viruses concurrently. One hundred and seventy specimens of blood were obtained for serological investigation and 255 throat swabs were taken for virus isolation.

Figure 1 represents the number of fresh cases of respiratory illness occurring during each week of the survey and the incidence of upper respiratory tract infection, laryngo-tracheo-bronchitis, bronchitis and pneumonia. It can be seen that at no time was the nursery completely free from respiratory illness and that four major outbreaks were observed. These occurred in November, late December, February and April.

Table 1 tabulates the results of complement-fixation tests carried out on sera obtained from each child at entry into the survey. It will be seen that apart from

Table 1. *Results of* C.F. *tests on sera taken at entry into the survey (number of cases with titre of eight or more)*

(No children had titre of eight or more to influenza A, B, parainfluenza 1 or respiratory syncytial virus.)

	Influenza		Parainfluenza		
	C	Sendai	2	3	Adeno
Babies	0	0	0	0	3
Infants	2	1	4	0	15
Toddlers	0	2	2	2	13
Total	2	3	6	2	31

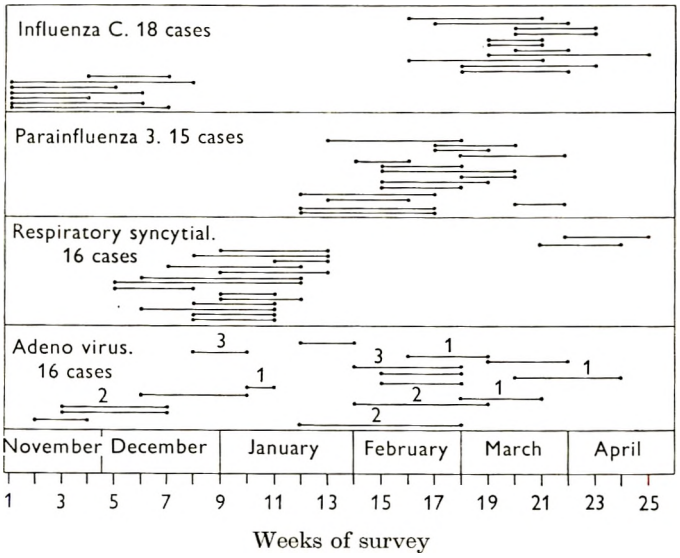

Fig. 2. The seasonal incidence of serologically proven virus infection throughout the survey (November 1963 to April 1964). ●—●, Period over which fourfold rise took place. Figures represent adenovirus types.

Table 2. *The incidence of virus infection in each disease category*

	No. of cases	No. of cases with virus infection	Cases with virus infection (%)
U.R.T.I.	76	28	37
L.T.B.	2	2	100
Bronchitis	23	13	56
Pneumonia	4	1	25
Total	105	44	42

adenovirus infection there was very little evidence of prior infection with any of the viruses tested.

Figure 2 presents the virus infections observed during the course of the 6 months. It will be seen that there were 16 cases of adenovirus infection, 16 cases of respira-

tory syncytial virus infection, 15 cases of parainfluenza 3 infection and 18 cases of influenza C infection; herpes simplex virus infection was encountered in only 2 cases. The adenovirus infections were distributed evenly throughout the time of the survey whereas the respiratory syncytial, parainfluenza 3 and influenza C infections occurred in well-defined outbreaks. The first three outbreaks of respiratory illness coincided with the outbreaks of infection with influenza C, respiratory syncytial, and parainfluenza 3 virus respectively and these were considered to be the aetiological agents responsible. The last outbreak of respiratory disease was not associated with any particular virus infection and occurred 1 month after the second influenza C outbreak. The total number of virus infections occurring in each disease category is shown in Table 2.

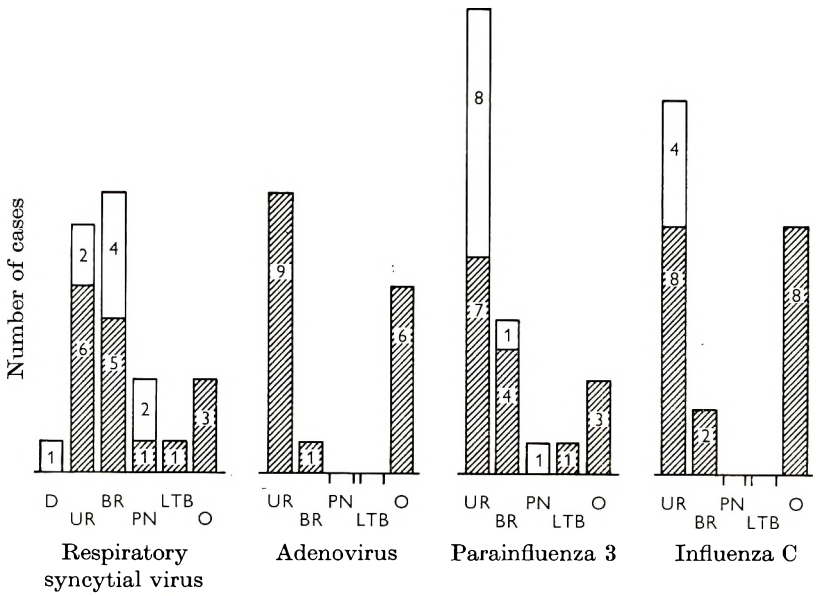

Fig. 3. The incidence and type of respiratory illness occurring in association with virus infection. ▨, Serological evidence of infection; □, clinical association only with virus outbreak. D, Died; UR, upper respiratory tract infection; BR, bronchitis; PN, pneumonia; LTB, laryngo-tracheo-bronchitis; O, no illness.

Influenza C outbreak

During the second week in November there was a sudden outbreak of mild upper respiratory disease amongst the infants and toddlers. Within 5 days 11 (55 %) of the 20 children at risk in these two groups developed a profuse mucoid nasal discharge. Of the children with symptoms, 7 developed a fourfold rise in complement-fixing antibody to influenza C antigen. Influenza C infection was encountered again in March when a further 11 cases were recognized—5 toddlers, 4 infants and 2 babies. Of these, 9 were children who had been admitted since the previous outbreak and 2 were children who had shown neither serological nor clinical evidence of infection during the previous outbreak. In the second outbreak only 3 children had clinical evidence of infection: 1 toddler and 1 infant developed

bronchitis and 1 baby had an upper respiratory tract infection. The analysis of the serological response to influenza C infection is shown in Table 3. The frequency with which the different categories of illness occurred is shown in Fig. 3.

Influenza C was not isolated in tissue culture during the course of the survey.

Table 3. *Serological response to influenza C in the different age-groups*

	No. of sera tested	Total with serological evidence of infection	No. with serological and clinical evidence of infection	No. showing clinical illness only
		First outbreak		
Babies	7	0	0	0
Infants	10	6	6	1
Toddlers	10	1	1	3
		Second outbreak		
Babies	6	2	1	0
Infants	13	4	1	0
Toddlers	11	5	1	0

Respiratory syncytial virus outbreak

During the 3 weeks from 18. xii. 63 to 8. i. 64 twenty (69 %) of the 29 children at risk became ill with respiratory tract illnesses and 14 (48 %) of the children at risk developed fourfold antibody rises to respiratory syncytial virus antigen. Three of the children who developed antibodies remained free from clinical evidence of infection and eight of the children who were ill had no increase in their respiratory syncytial virus antibody (convalescent serum was not obtained from one child). The type of illness varied with the age of the child. Seven of the toddlers developed upper respiratory tract illnesses only and one developed bronchitis. Five of the infants developed bronchitis, one developed pneumonia (a child who was admitted during the outbreak) and one died. This fatal case was a 17-month-old female child with no previous history of respiratory illness. She developed a mucoid nasal discharge and became irritable on 19. xii. 63. Early on the morning of 20. xii. 63 she suddenly became dyspnoeic and cyanosed and died before the arrival of the nursery doctor. She was apyrexial throughout her illness. A specimen of serum and a throat swab had been taken a few days before death but no other specimens were obtained. No evidence of virus infection was found on culture of the pharyngeal swab or from complement-fixation tests. Autopsy was not carried out.

Three of the babies developed severe bronchitis, two had pneumonia and two remained well. The serological response to infection with respiratory syncytial virus appeared to vary among the children according to age as can be seen from Table 4. It was considered that the illnesses among the babies were due to infection with respiratory syncytial virus despite the lack of serological proof as their illnesses were so closely related in time and clinically to the outbreak of respiratory syncytial virus infection among the older children.

Two children developed fourfold antibody rises to respiratory syncytial virus

during April. One, an infant who had had pneumonia during the previous outbreak but no serological evidence of infection, developed laryngo-tracheo-bronchitis in association with a fourfold rise in titre, and the other, a toddler who was admitted after the outbreak, developed a fourfold antibody rise to respiratory syncytial virus with an upper respiratory tract infection.

Table 4 shows the analysis of serological response to respiratory syncytial virus, and Fig. 3 an analysis of the types of diseases encountered.

Thirty-eight pharyngeal swabs were taken from the children during the outbreak. No respiratory syncytial virus was isolated in tissue culture but adenoviruses were isolated from three swabs (types 1, 3 and 5) and one unidentified cytopathic agent was isolated in human embryo lung cells.

Table 4. *Serological response to respiratory syncytial virus in different age-groups*

(Figures in parentheses represent the two sporadic cases.)

	No. of sera tested	Total with serological evidence of infection	No. with serological and clinical evidence of infection	No. showing clinical illness only
Babies	7	1	1	4
Infants	10 (+1)	5 (+1)	4 (+1)	3
Toddlers	11 (+1)	8 (+1)	6 (+1)	2

Table 5. *Serological response to parainfluenza* 3 *in different age-groups*

	No. of sera tested	Total with serological evidence of infection	No. with serological and clinical evidence of infection	No. showing clinical illness only
Babies	7	4	4	3
Infants	8	5	5	2
Toddlers	9	6	3	1

Parainfluenza 3 *outbreak*

Between 6. ii. 64 and 24. ii. 64 twenty-two (71 %) of the children at risk became ill with respiratory symptoms. Fifteen children (48 %) developed a fourfold rise to parainfluenza 3 over this period and parainfluenza 3 virus was isolated on two occasions. Three of the children who developed antibodies had no respiratory illness, six children who did not develop antibodies had respiratory illnesses at this time and three children had neither respiratory illnesses nor a rise in antibody. Seven children had no convalescent serum tested and of these four had respiratory illnesses. The serological response in the three age-groups was more uniform during this outbreak than in the respiratory syncytial virus outbreak as is shown in Table 5.

The types of illness encountered during this outbreak are shown in Fig. 3. It was

noticed that the severity of illness was greater among the babies. One baby developed bronchopneumonia, 1 laryngo-tracheo-bronchitis which required hospital admission, 3 bronchitis and 3 developed upper respiratory tract illnesses. Of the 23 infants and toddlers, 12 had upper respiratory tract illnesses and 2 had bronchitis.

Adenovirus infection

During the survey adenoviruses were isolated from throat swabs on 21 occasions (adenovirus type 1, 6 cases; type 2, 8 cases; type 3, 6 cases, type 5, 1 case). On 10 occasions the isolation was accompanied by a fourfold rise in neutralizing antibody titre to the virus isolated.

On a further six occasions a fourfold rise in complement-fixing antibody for adenovirus unaccompanied by virus isolation occurred and the type of adenovirus responsible was not ascertained. These sixteen proven infections are shown in Fig. 2 where it will be seen that both the typed and untyped infections occurred sporadically throughout the period of study.

The majority of adenovirus infections were associated with either upper respiratory tract infection (9 cases) or no clinical illness (6 cases). One case of bronchitis in association with adenovirus infection occurred.

A small outbreak of conjunctivitis occurred in the nursery during April, but adenovirus was not isolated from throat or conjunctival swabs from these children.

DISCUSSION

In this Edinburgh nursery two distinct patterns of infection emerged. The adenovirus infections were endemic in the nursery and occurred throughout the period of study. The respiratory syncytial virus, parainfluenza 3 virus and influenza C virus infections were epidemic in character, occurring in well demarcated outbreaks. This type of pattern is similar to that found by Sutton (1962) in his survey in which he found outbreaks of parainfluenza 3 virus infection and influenza A virus infection occurring against an endemic pattern of adenovirus infection. This difference in epidemiological pattern appears to be the result of previously acquired immunity as 31 (76%) of the 41 children showed serological evidence of previous adenovirus infection whereas only 2 children showed evidence of prior parainfluenza 3 infection, 2 showed evidence of influenza C infection and none showed evidence of respiratory syncytial virus infection. It was impossible to obtain sufficient sera from the children for neutralization tests against the prevalent adenovirus types and therefore we were unable to ascertain the type of adenovirus responsible for the previous infections.

No distinctive clinical picture for any of the infections was evident either on clinical examination at the time of infection or on retrospective analysis of the symptoms and clinical signs. It was apparent, however, that infection by respiratory syncytial virus and parainfluenza 3 virus resulted in a much more severe illness, particularly among the infants, than did influenza C virus or adenovirus infection. Respiratory syncytial virus is known to be causally related to epidemic bronchio-

litis in infants (Sandiford & Spencer, 1962) but in the present outbreak no cases of bronchiolitis were recognized.

The most severe cases of respiratory syncytial virus infection showed radiological evidence of bronchopneumonic consolidation and the others developed acute bronchitis, upper respiratory tract illness or showed no evidence of infection. Unlike parainfluenza virus 1 and 2 which are established causes of laryngo-tracheo-bronchitis in young children (Parrott *et al.* 1963), parainfluenza 3 has been found in association with a wide range of respiratory disease from severe pneumonia to mild upper respiratory tract infection (Chanock *et al.* 1963). This pattern was seen in the present outbreak where illness ranged from severe bronchitis in the younger age-groups to asymptomatic infection in the older children and included one case of laryngo-tracheo-bronchitis severe enough to require hospitalization.

It is surprising to find two clear-cut outbreaks of influenza C in our survey as evidence of infection with this virus is unusual in children. The apparent infrequency of influenza C infection in children may be due to the mild illness it produces which results in few cases being investigated virologically.

The most significant factor affecting the severity of the illness appears to be host resistance as reflected by the age of the child. No serious respiratory illness occurred in any child over the age of 9 months whereas seven of the infants, one on two occasions, required hospital admission for severe respiratory illness.

In a compact community such as the nursery where all the occupants were equally at risk it was surprising to find that during the respiratory syncytial virus outbreak only 48% of the children developed antibody. This is much lower than the rate of infection found by Kapikian *et al.* (1961) in the Washington Children's Village outbreak where 78% of the population at risk developed a fourfold rise in antibody titre. In the Washington Children's Village all the children were over 6 months of age and for a direct comparison it is necessary to correct our figures for age by excluding all children less than 6 months old. When this is done 66% of the children are found to have developed a fourfold rise in antibody titre.

Despite the lack of serological evidence among the babies it is highly probable that they were infected by respiratory syncytial virus at this time as five out of seven of them developed severe respiratory illness concurrently with the serologically proven respiratory syncytial virus outbreak among the older children. Thus it is apparent that the antibody response to respiratory syncytial virus infection among the babies was much poorer than in the older children. This difference may be expressed as fourfold rises in antibody titre to respiratory syncytial virus per child: babies (< 7 months), 0·14 fourfold responses per child; older children (7 months and over), 0·63 fourfold responses per child.

This is in contrast with the antibody response during the parainfluenza 3 outbreak where the following fourfold antibody responses to parainfluenza 3 virus occurred: babies (< 7 months), 0·57 fourfold responses per child; older children (7 months and over), 0·64 fourfold responses per child.

These findings suggest that the poor antibody response to respiratory syncytial

virus in the infants was not due to the inability of the infants to produce complement-fixing antibody to virus infection but rather to a failure to produce antibody to respiratory syncytial virus in particular. This apparently inadequate antibody response in infants to infection with respiratory syncytial virus may be of significance in the pathogenesis of the very severe respiratory illnesses characteristic of infection with this virus in infants (Crone, Heycock, Noble & Patton, 1964).

The high incidence of respiratory infections in children's homes is a major problem. The greatest danger is to children of less than 7 months and it is important therefore to take special measures to protect communities of infants from the introduction and spread of viral pathogens.

SUMMARY

Forty-one children in an Edinburgh nursery were observed for evidence of respiratory illness from November 1963 to April 1964. During this period serial specimens of serum from these children were examined for virus antibodies and serial throat swabs were investigated for the presence of virus. Well-defined outbreaks of respiratory illness occurred and could be associated with respiratory syncytial virus, parainfluenza 3 virus and influenza C virus infection. Infection with adenovirus followed a more endemic pattern. The antibody response in infants to respiratory syncytial virus as measured by the standard complement-fixation test was found to be much poorer than the response to parainfluenza 3 virus. It is suggested that this may play a part in the severity of respiratory syncytial virus infection in infants.

We are grateful to the Children's Department of Edinburgh Corporation and to the Matron of St Katharine's Home for their permission to examine the children and for their help in carrying out the work. We would also like to thank Prof. Robert Cruickshank, Prof. John Crofton, Dr R. H. A. Swain, Dr A. T. Wallace and Dr A. K. Hornsleth for their advice and encouragement. We are also grateful to Mrs Hazel Cross, Mr H. W. Moncreiff and Mr R. S. Anderson for technical assistance and to Miss Joyce Holywell for secretarial assistance. The Scottish Hospitals Endowments Research Trust provided generous grants to support this work.

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Experiments on the communion cup

By BETTY C. HOBBS, JILL A. KNOWLDEN and ANNE WHITE
Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

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The work described in this paper was undertaken as the result of a request to the Medical Research Council for information on the danger of disease being transmitted by a common communion cup.

Besides the more direct approach, observations were made on the value of a cloth or purificator for wiping the rim of the cup after each communicant, and of rotation of the cup so that each communicant has a fresh portion of the rim presented to him.

A silver communion cup or chalice was used for these experiments with a bowl 12 cm. in diameter gilded on the inner surface. The cup was not polished in the course of the experiment, but merely washed well in warm running water and dried after each experiment. Sacramental wine was used; it was not diluted, because inquiries concerning this practice showed such diversity in custom in individual churches, some using the wine undiluted, that it seemed preferable to use the neat wine rather than to adopt an arbitrary concentration. Chemical analysis of the wine showed that the alcohol content was approximately 14·5 %.

Part 1. Survival of organisms in saliva, wine and Ringer's solution on the chalice surface

Methods

A. *Numbers of organisms recovered from the chalice after drinking*

(i) Volunteers were asked to drink wine from the chalice. On the first occasion after drinking, a small area of the rim, 4 cm. wide by 2 cm. in depth, was swabbed on both the inner and outer surfaces with two calcium alginate swabs (Higgins, 1950). The first swab was moistened in 9 ml. of quarter-strength Ringer's solution, rubbed over the appropriate area and then broken off into the Ringer's solution; the second swab was used to remove excess moisture and broken off into the same Ringer's solution. One ml. of a 10 % solution of sodium hexametaphosphate was added and the solution shaken gently for a few minutes to dissolve the swabs; this suspension was used for making plate counts. The experiment was repeated for each person.

On the second occasion the drinking area was wiped gently with a linen cloth after each person in order to imitate the use of the purificator in church. The same area of the chalice was then swabbed as before on both inner and outer surfaces.

Counts were made either from drops of known volume dried on the surface of

blood agar (Miles & Misra, 1938) or, when experience suggested that few bacteria would be present, in pour plates of nutrient agar containing 10% horse serum.

The experiment was repeated with several volunteers and in some instances with the same person on different occasions.

(ii) A similar experiment was carried out with six volunteers who drank from the communion cup in turn at approximately 5 sec. intervals. The cup was half filled with wine at the beginning of the experiment. A few drops of the wine were dropped onto the surface of blood agar as a negative control for the wine; this was repeated on a number of occasions with negative results. The inner and outer surfaces of the chalice were swabbed at the beginning of the experiment to determine the number of bacteria present on the cup initially. The communion cup was passed around the six volunteers four times, each time with a different procedure, and after each round, which took approximately 40 sec., the whole of the inner and outer drinking surfaces were swabbed to a depth of 2 cm. from the edge.

The variations in the procedure were carried out in the following order:

(*a*) All drinking from the same place.

(*b*) Rotating the cup so that each person drank from a different place.

(*c*) Drinking from the same place but wiping gently with a linen cloth after each person.

(*d*) Rotating the cup so that each person drank from a different place which was wiped after each person. This experiment was repeated on six different occasions.

B. *The survival of organisms present in saliva deposited on the surface of the chalice*

Saliva was collected in a sterile test-tube, and by means of a standard size platinum loop a small quantity was deposited on the dry chalice over an area approximately 4 cm. wide by 2 cm. in depth; this area was swabbed, according to the procedure described in section A, after different intervals of time up to 3 min. This was repeated for the inner and outer surfaces of the chalice.

C. *The survival of organisms suspended in wine and in Ringer's solution and deposited on the surfaces of the chalice*

Five cultures were used, two strains of *Staphylococcus aureus* F 6186 (phage type 6/7/42E/47/54/75) and St. 61.17004 (phage type 80/81), *Escherichia coli* type 1 and *Streptococcus pyogenes* (R.61.4139). A suspension of approximately 10^8 organisms per ml. was made from each in saline. One ml. of each suspension was added to 9 ml. of wine and 1 ml. to 9 ml. of Ringer's solution. A loopful of these suspensions was placed on the surface of the chalice as described in the previous experiment and each region was swabbed after different periods of time.

RESULTS

A. *Numbers of organisms recovered from the chalice surface after drinking*

The results are given in Tables 1 and 2. Table 1 shows the variability of the results obtained from one person and also that, although the wiping cloth usually removed a proportion of the organisms, the extent of the removal was variable.

Table 1. *Experiments to show the number of organisms deposited by individual persons on the chalice during drinking and showing the effect of wiping on the numbers of organisms recovered*

	Without wiping	With wiping
1A	1260	320
B	400	100
2A	20	None
B	None	None
3A	30	15
B	40	None
4A	2650	15
B	2730	100
5	20	20
6	20	100
7	25	None

Table 2. *Effect of wiping and of rotating the chalice on the numbers of organisms recovered from the drinking surface (six persons)*

	Experiment no.					
	1	2	3	4	5	6
Control swab from chalice	None	20	22	63	10	45
(1) Drinking from same place	485	2700	1700	7820	9200	1670
(2) Drinking from different places	910	3020	3320	7840	42900	5200
(3) Drinking from same place and wiping	215	125	320	790	1730	2300
(4) Drinking from different places and wiping	765	305	465	920	9440	80

Table 3. *Survival of organisms suspended in saliva when deposited on inner and outer surfaces of the dry chalice*

Time (min.)	Experiment 1		Experiment 2		Experiment 3	
	Outside	Inside	Outside	Inside	Outside	Inside
0	122,000	34,000	55,000	220,000	35,000	90,000
½	48,000	34,000	70,000	115,000	29,000	70,000
1	130,000	13,000	60,000	90,000	2,000	40,000
2	70,000	1,000	35,000	115,000	15,000	35,000
3	1,000	12,000	10,000	80,000	40,000	38,000

Table 2 shows the more consistent results obtained when six persons drank from the chalice, with and without wiping. More organisms were recovered from the chalice after rotating the cup to present a fresh surface for each participant

than when all were drinking from the same place. This may be explained by the assumption that when each person took the wine from the same region some bacteria were removed and some others were deposited, whilst when the cup was rotated all organisms deposited by preceding participants remained, so that each person added a complement to the total. There was considerable variation in results from person to person as would be expected. No organisms were ever isolated from the wine itself, even after six persons had taken four sips each.

B. *Determination of the survival of various organisms present in saliva deposited on the surfaces of the dry chalice*

The results are shown in Table 3. In most instances there was a small reduction in bacterial numbers at the end of the 3 min. test. It appears that short intervals of time cannot be expected to sterilize the surface of the chalice or even to decrease the possibility of contracting disease unless certain organisms are exceptionally sensitive to these conditions. In fact the time elapsing between communicants may be as short as 3 sec.

Table 4. *Survival of* Staphylococcus aureus *suspended in wine and Ringer's solution and deposited on inner and outer surfaces of the chalice*

	F 6186				St. 61.17004			
	Outside		Inside		Outside		Inside	
Time (min.)	Wine	Ringer's solution	Wine	Ringer's solution	Wine	Ringer's solution	Wine	Ringer's solution
0	7830	3600	1020	1670	400	3190	5200	2170
½	4560	1250	330	210	1320	1650	1720	3370
1	4580	1790	850	490	1370	1390	740	3850
2	1180	3860	170	160	270	660	1620	1510
3	2450	1950	220	200	170	220	580	1440

Table 5. *Survival of* Escherichia coli I *suspended in wine and Ringer's solution on inner and outer surfaces of the chalice*

	Inside		Inside		Outside	
Time (min.)	Wine	Ringer's solution	Wine	Ringer's solution	Wine	Ringer's solution
0	530	720	230	2150	2970	7260
½	70	990	130	1400	1620	3820
1	50	780	30	290	1140	3700
2	20	340	20	890	700	2860
3	0	1110	60	780	80	2570

C. *Determination of the survival of various organisms, suspended in wine and in Ringer's solution, on surfaces of the chalice*

The results are shown in Tables 4, 5 and 6. There was always some reduction in the numbers of organisms recovered after 3 min., but it was rarely as great as 90 %.

Experiments with *Staph. aureus* showed no difference between results obtained with the organisms suspended in wine or in Ringer's solution. However, experiments with *Esch. coli* and *Strep. pyogenes* showed that bacterial survival was greater in Ringer's solution than in wine.

Table 6. *Survival of* Streptococcus pyogenes *suspended in wine and Ringer's solution and deposited on inner and outer surfaces of the chalice*

	Inside		Outside	
Time (min.)	Wine	Ringer's solution	Wine	Ringer's solution
0	20	270	0	1330
½	0	225	45	1200
1	0	115	5	495
2	0	60	0	80
3	0	0	5	10

Part 2. Effect of wine and silver on various organisms

A series of fifteen experiments was carried out to investigate the effect of the wine and the silver and a combination of both factors on *Esch. coli* (026), *Staph. aureus* F 6186, *Serratia marcescens* NCTC 9940 and *Strep. pyogenes* (R.61.4139).

Initial inocula were approximately 100,000–200,000 organisms/ml. in 10 ml. wine held in sterile Universal containers with or without a lining of thin sheet silver. Similar receptacles with suspensions of the same organisms in 10 ml. quarter-strength Ringer's solution were used as controls for each experiment.

Exposure times varied from 15 sec. (the initial count) to 60 min. with sampling intervals of 1, 2 or 4 min. in different experiments.

The effect of exposure was observed either by noting growth or sterility in Todd Hewitt broth inoculated with a loopful of the experimental suspensions, or more exactly by pour plate counts.

In four experiments *Esch. coli* in wine was killed in 12 but not 10 min. (two experiments) and 14 but not 12 min. (two experiments); and, in two experiments out of three, silver reduced the killing time by approximately 1 min. in one experiment and 4 min. in the other. In the same four experiments *Staph. aureus* in wine was killed in 20 but not 18 min., in 12 but not 8 min., and in 18 but not 16 min. (two experiments). The effect of silver was to reduce the killing time by 10 min. in one experiment, by 4 min. in two experiments, and not at all in one experiment.

In two experiments *Serratia marcescens* in wine was killed in 16 but not 14 min., when silver had no effect, and in 12 but not 10 min. when silver reduced the time by 2 min.

In three experiments with *Strep. pyogenes* in wine, the organism was killed in 4 but not 2 min. in one experiment, when the time was reduced to 2 but not 1 min. by silver, and in 1½ but not 1 min. with no reduction by silver in two experiments. Examples of counts obtained in some of these experiments are given in Table 7.

In a series of three experiments wine was reinoculated at intervals with the test organisms in an effort to simulate the continuous use of the common chalice. The same cup of wine could be used for a varying number of people depending on the size of the cup. It would take approximately 50 sec. to serve 10 persons, and perhaps 10 sec. would be used to return to the beginning of the row of people. It is likely that as many as seventy people could thus partake of the same wine in approximately 7–10 min. and during this time fresh organisms would be added to the wine. Refilling of the cup would dilute the residual wine and organisms, but with 200 communicants, for example, there could be a continuous inoculation process for 20 min. or so. This presupposes the carriage of similar organisms by a number of people which would perhaps occur only at epidemic times. These experiments will now be described.

Table 7. *Effect of wine and wine and silver together on various organisms*

	Count per ml. (2 days at 37° C.)							
	Esch. coli		*Staph. aureus*		*S. marcescens*		*Strep. pyogenes*	
Time	Wine	Wine and silver	Wine	Wine and silver	Wine	Wine and silver	Wine	Wine and silver
15 sec.	90,000	61,000	67,000	76,000	180,000	190,000	3,700	21,000
30 sec.	—	—	—	—	—	—	1,800	1,700
45 sec.	—	—	—	—	—	—	120	850
1 min.	—	—	—	—	—	—	90	120
1½ min.	—	—	—	—	—	—	< 5	< 5
2 min.	—	—	—	—	—	—	< 5	< 5
2½ min.	—	—	—	—	—	—	< 5	< 5
4 min.	7,900	6,800	14,000	3,800	160,000	1,200	—	—
6 min.	2,400	1,300	620	110	25,000	—	—	—
8 min.	580	70	80	70	8,900	120	—	—
10 min.	20	< 5	10	50	700	< 5	—	—
12 min.	10	< 5	< 5	5	< 5	< 5	—	—
14 min.	< 5	< 5	15	< 5	< 5	< 5	—	—
16 min.	—	—	5	< 5	< 5	< 5	—	—
18 min.	—	—	< 5	< 5	—	—	—	—

In two experiments suspensions of *Esch. coli* and of *Staph. aureus* in 10 ml. quantities of wine held in silver-lined universal containers were continually reinoculated with the same quantity of a suspension of the relevant organism in wine; the additions were made at 1, 3, 5 and 7 min. Samples were removed for counts at 2 min. intervals from 2–24 min. The original count taken at 15 sec. was 30,000/ml. for *Esch. coli* and 45,000/ml. for *Staph. aureus*, and the survival times reckoned from the first addition of the wine suspension to the universal containers were 14 but not 16 min. for *Esch. coli* and 18 but not 20 min. for *Staph. aureus*.

In a third experiment the same procedure of reinoculation was followed using *Strep. pyogenes*. Starting with a count of 4700/ml. at 15 sec. and reinoculating at 1, 1½, 2 and 2½ min. the streptococci survived for 3 but not 3½ min.

In a further experiment with *Strep. pyogenes* it was observed that, with an inoculum of approximately 670,000/ml., streptococci suspended in neat wine

were destroyed in 3 min., but in wine diluted 1/2 to 1/256 with quarter-strength Ringer's solution the destruction time was lengthened but the actual time beyond 3 min. was not ascertained. In practice the dilution of wine with water varies according to the practice of the incumbent but it is likely that a few drops only of water will be added to any volume of wine.

The inhibitory effect of silver ions was demonstrated directly with *Esch. coli* but not with *Staph. aureus*. Two methods were used:

(*a*) 1 cm.^{2} portions of silver foil and glass (control) were introduced into an inoculated pour plate and incubated for 48 hr. at 37° C. Colonies of both organisms growing beneath both silver and glass were reduced in size but there was a noticeable reduction in numbers of colonies of *Esch. coli* only beneath the silver; the effect was most apparent in pour plates with small inocula.

(*b*) Nutrient agar plates were inoculated by flooding the surface with suspensions of *Esch. coli* or *Staph. aureus*; pieces of silver foil and glass were placed on the surface. On the *Esch. coli* plates the silver strips but not the glass were surrounded by a zone of incomplete inhibition about 0·5 mm. wide; also the growth under the silver strips was more scanty than that beneath the glass strips.

An experiment in which silver and glass strips were directly contaminated with drops of wine containing *Esch. coli* and *Staph. aureus*, and were inoculated into Todd Hewitt broth after various exposure times, was unsatisfactory. The results were irregular, with survival times for *Esch. coli* up to 20 min. on silver and 24 min. on glass and for *Staph. aureus* up to 24 min. on silver and 30 min. on glass.

The results of these experiments confirmed the findings described in Part I, namely that, with the organisms tested, the effect of the wine in destroying bacterial cells deposited on a chalice would not be fast enough, even for *Strep. pyogenes*, to take place during the rapid passage of the chalice from person to person. They show too that, although silver may enhance the destruction of bacteria by wine, the effect is too small to be of value.

DISCUSSION

The possible spread of infectious disease by the common communion cup has been under discussion for many years. Attention was concentrated on the subject in the United States of America by the observations of Forbes in 1894 and Anders in 1897.

According to Anders (1897), Forbes reported to the Rochester Pathologic Society 'in the dregs of the ordinary cup, contamination from both the mouth and clothing; from the former, epithelial cells, mucus, and various bacteria and spores; from the latter, fibrous material. Control experiments showed the unused wine to be practically sterile.' Without much more information than is given it would be difficult to assess the significance of these findings. The origin of the epithelial cells could have been the skin, and the sporing bacilli and fibrous material could have come from dust in the air.

Anders (1897) himself records very briefly the results of observations he made in 1894 with the assistance of Dr Furbush. Without giving any technical details

he states that he found tubercle bacilli in two out of five specimens of dregs from the communion cup, besides pus cells, oral epithelial cells and 'pus staphylococci'. Here again, it is difficult to assess the significance of the results. Acid-fast bacilli are common in dust and in water from metal taps and could not be distinguished microscopically from tubercle bacilli. It is improbable that the bacilli were cultured, and therefore the statement that tubercle bacilli were found must be accepted with the greatest reserve. No mention is made of how the 'pus staphylococci' were disinguished from ordinary staphylococci and micrococci that are common on the skin and in the mouth, nor how the epithelial cells were shown to be of oral origin.

The combined effect of the observations, and still more the pleading, of Forbes and Anders was to promote a lively interest in the use of individual cups for communion. Anders presses strongly for their adoption. He doubts whether at the Last Supper only one cup was used and whether it was, in fact, passed round. There is no explicit statement in the Gospels to this effect, and among Jews at the time of Christ individual cups are said to have been the rule.

The next series of observations appears to have been in 1925 by Page in the United States. According to Burrows & Hemmens (1943), Page took cultures from the rim of the chalice after use, and dropped the purificator (the cloth used for wiping the cup) into broth. Mice and guinea-pigs were inoculated with the cultures. Numerous organisms were isolated, mainly sporing and non-sporing bacilli, staphylococci, yellow cocci, white cocci and other cocci. Of the 18 mice inoculated 5 died, and of the 19 guinea-pigs inoculated 8 died. As Burrows & Hemmens point out, there is little evidence to show that these organisms were of salivary origin; it is more probable that they came mainly from dust.

Burrows & Hemmens themselves performed a number of experiments on both the communion cup and the purificator. There is no need to describe them in detail. Their main findings were that *Strep. pyogenes*, when suspended in filtered saliva, died off rapidly in contact with the silver chalice, a high proportion being dead within 2 min.; that 80–90 % of organisms were removed by the purificator; that under the most favourable conditions for transference only about 0·001 % of organisms were transmitted from the saliva of one person to the mouth of another; and that when conditions approximated to those in actual practice no transmission could be detected. Their general conclusions are that the communion cup cannot be regarded as an important vector of disease.

Rather different results were obtained by Gregory, Carpenter & Bending (1963) in Canada. They found that contact with the silver chalice had no apparent effect on cells of *Strep. pyogenes* suspended in saliva within 60 min., though when unprotected by saliva they died in the wine itself within 2–3 min. The difference between these results and those of Burrows & Hemmens was probably due to the use by Burrows & Hemmens of filtered saliva, which contained far less protective protein than raw saliva. Gregory, Carpenter & Bending studied the rate of passage of the chalice during communion services, and found that the average time between successive communicants was about 5 sec. From the test cup after a simulated communion service they isolated species of *Bacillus*, *Micro-*

coccus, *Neisseria*, *Staphylococcus* and *Streptococcus*. They point out that 5 sec. is far too short a time to effect destruction of these organisms and therefore conclude that the common communion cup and its contents could serve as vehicles for the rapid transmission of micro-organisms.

The results of our own experiments lie rather between those of Burrows & Hemmens and those of Gregory *et al.* They showed that the organisms in saliva, when in contact with the inner surface of the chalice, decreased in numbers within 3 min., and that *Strep. pyogenes*, when suspended in wine, perished on the inside of the chalice in between 2 and 3 min. Like Burrows & Hemmens, we found that the purificator removed about 90 % of the organisms on the rim of the cup. Rotation of the cup had less effect than was expected, probably because the time of complete rotation is too short to allow destruction of the organisms deposited on the rim, and partly because each person deposits traces of saliva of his own which replace those of the communicant before him. Rotation, in fact, benefits only the communicants in the first round of the cup. The rest are exposed to much the same degree of contamination as when the cup is not rotated.

On technical grounds it must be admitted that during an ordinary communion service the rim of the chalice inevitably becomes contaminated with the saliva of the participants; that the organisms present in the saliva of one person are transmitted to the next one in turn; that the combined effect of the wine and the silver of the chalice is insufficiently rapid to ensure the destruction of these organisms in the short interval between successive communicants; and that therefore the common communion cup must serve as a vehicle for the transmission of infective organisms.

After such an admission we must ask ourselves what is the risk of contracting infectious disease in this way. There are two main reasons for assuming that it is not great.

In the first place the number of bacteria on the lips, though varying greatly with different persons, tends to be small and the chance of pathogenic bacteria being among them may be very low indeed. When pathogens are present, the numbers are probably so small that the risk of ingesting them may be negligible. The body can deal with very small numbers of most pathogenic organisms, so that even if a few were ingested they would be unlikely to give rise to disease.

The second reason is an even stronger one. It is that the organisms which infect by the mouth, such as the typhoid and the dysentery bacilli, are not likely to be found on the lips. They are excreted in the faeces or urine and contaminate the fingers but not the lips. On the other hand it is thought that the pathogenic organisms that may be found on the lips are unlikely to infect by the mouth. Micro-organisms including viruses which are mainly responsible for respiratory disease gain access to the body through the nose, and possibly the conjunctiva, but do not as a rule, unless present in large numbers, give rise to infection by the mouth, although this may happen. It is hoped that persons with acute infection, particularly of the throat, will not communicate. But salivary carriage of *Strep. pyogenes* may occur in patients with or recovering from acute throat infection but fit enough to go to church, and the streptococcal count in the saliva may be quite

high (Hamburger, 1944). An additional point may be made, namely that many of the more easily transmissible diseases are diseases of childhood, and that in the Church of England few young children are communicants, and they are becoming fewer.

It is conceivable, of course, that a patient suffering from a syphilitic sore on the lip or in the mouth might contaminate the rim of the chalice and pass on infection in this way. The chances of this occurring are remote, but if such a person wished to partake of Holy Communion it is unlikely that the officiating priest would recognize the lesion.

Disease from tubercle bacilli follows the inhalation of infected particles. It may occur by mouth but for an infective dose the organisms must be present in large numbers and ingested over a long period of time.

It is difficult to assess the significance of the common communion cup in the transmission of infectious disease. The difficulty of obtaining definite proof, one way or the other, is almost insuperable. There are in any gathering, such as that in a church, other commoner and probably far more effective ways in which respiratory and alimentary infections can be spread. Unless quite unusual circumstances prevailed, it would be almost impossible to incriminate the communion cup.

What risk there is of transmission could be greatly diminished by the use of a purificator in between each communicant, or by the use of individual cups as practiced by many Protestant denominations; though we realize that not all officiating priests would agree to these practices. The risk could be removed also by substituting the method of intinction in which the bread is dipped within the wine so that both elements in the sacrament are given simultaneously. Clergy visiting the sick administer the elements in this way but there might be practical difficulties in adapting the method for large numbers of people. No objection was raised to the method of intinction at the Lambeth Conference of 1945 which, under Resolution 118, recommended that any part of the Anglican Communion should by provincial regulation have liberty to sanction administration by intinction as an optional alternative to the traditional method. However, the recent Liturgical Commission considering the new communion service emphasized very strongly that consecrated bread from one loaf and consecrated wine from one chalice should be delivered to the people (Liturgical Commission, 1965).

SUMMARY

Experiments were made to find out whether the common communion cup is likely to serve as a vehicle for the transmission of infection.

A silver chalice and sacramental wine containing 14·5% of alcohol were used.

Observations with volunteers showed that the number of organisms deposited on the rim of the chalice varied from person to person, but was usually quite small—less than 100.

Rotation of the cup was of no benefit except to those partaking during the first round, since the saliva deposited on the rim by each person in turn remained

to contaminate the cup during the second round, and the combined effect of the alcohol and the silver of the chalice was not rapid enough to destroy the contaminating organisms before rotation of the cup was completed.

On the other hand the use of a linen cloth or purificator led to a diminution of about 90% in the bacterial count of the cup.

Organisms in saliva deposited on the interior of the dry chalice suffered some diminution in numbers within 8 min., presumably as the result of the disinfectant action of the silver, but the effect was too small to be of significance.

When suspended in wine and deposited on the internal surface of the chalice *Escherichia coli* suffered a substantial reduction within 3 min., *Streptococcus pyogenes* was destroyed completely; but *Staphylococcus aureus* was affected to a much less extent.

Various experiments designed to measure the disinfectant action of wine, and of silver and wine together, showed that the augmenting effect of silver on the disinfectant action of the alcohol was quite small. *Strep. pyogenes* proved to be far more sensitive to alcohol than *Esch. coli*, *Staph. aureus* and *Serratia marcescens*. Under the conditions of the experiment these last three organisms were not destroyed for 10–12 min., whereas *Strep. pyogenes* perished within $1\frac{1}{2}$ min.

The results of our work are in general agreement with those of previous workers, and show that the organisms deposited on the rim of the communion cup are not destroyed within the short time—5 sec. as an average—elapsing between the partaking of the sacrament by each successive communicant.

It must therefore be admitted that the common communion cup may serve as a means of transmitting infection. Reasons are given, however, for believing that the risk of transmission is very small, and probably much smaller than that of contracting infection by other methods in any gathering of people.

Such risk as there is could be greatly diminished by the use of a purificator for wiping the cup between each communicant, and could be abolished completely by substituting individual cups or by the practice of intinction.

We thank Sir Graham Wilson for the opportunity of doing this investigation and also for his guidance throughout the work and compilation of the paper. We are grateful also to F. Osborne and Co. Ltd. for the loan of the Chalice and also to the Government Chemist and Thomas McLachlan and Partners who checked the alcohol content of the wine.

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Thermal inactivation of diphtheria toxoid following drying by sublimation *in vacuo*

By VLADIMIR DAMJANOVIC* and MARIJA IOVICIC
Institute for Sera and Vaccines 'Torlak', Beograd, Yugoslavia

The denaturation of proteins consists of a physical rearrangement of the peptide molecule; primarily an unfolding of peptide chains (Putnam, 1953; Haurowitz, 1963). If denaturation is heat induced, the peptide chains unfold only if water flows into the spaces between the chains (Haurowitz, 1963). Denaturation can be minimized if the water content of the protein is reduced by sublimation *in vacuo* prior to heating (Greaves, 1946; Greiff & Pinkerton, 1954; Greaves, 1960). In this case, stability of the protein at increased temperatures is a function of the amounts of water in the dried product (Greaves, 1960). Because residual moisture is not the only factor affecting the stability of the dried product, the stability of dried antisera, toxins, virus, etc., is based frequently on the measurement of a biologic parameter and not on a direct analysis of residual moisture (Jerne & Perry, 1956; Fisek, 1959; Neumann, 1960; Greiff & Rightsel, 1965). The present study reports the effects of elevated temperatures on the stability of dried diphtheria toxoid, the stability of dried diphtheria toxoid when in combination with tetanus toxoid, and the stability of diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine.

MATERIALS AND METHODS

A purified preparation of diphtheria toxoid was used in all investigations (Holt, 1950). Activities of diphtheria toxoid were measured by L_f values, that amount of toxoid which gives most rapid flocculation with one standard unit of antitoxin, and K_f values, the time required in a flocculation test (Ramon technique) for particles to become visible to the unaided eye (Boyd, 1956). In those cases where weakly combining toxoid was present, flocculation was aided by the addition of an active diphtheria toxin (Regamey, 1957). When diphtheria toxoid was a component of diphtheria–tetanus–pertussis vaccine, the flocculation test was carried out after centrifugation to remove bacteria.

The concentration of diphtheria toxoid component, alone or in combination with tetanus toxoid, was adjusted so that each 1 ml. sample dried by sublimation *in vacuo* contained 350 L_f units. For testing, each dried preparation was rehydrated with 10 ml. of a concentration of sodium chloride sufficient to result in an isotonic solution and an activity of 35 L_f/ml. In the case of diphtheria toxoid in combination with tetanus toxoid and pertussis vaccine, the concentration of the former was adjusted so that 2 ml. of the suspension contained 290 L_f. Two ml. volumes of the

* Present address: Department of Pathology, Marquette University School of Medicine, Milwaukee, Wisconsin, U.S.A.

suspension above were dried by sublimation *in vacuo* and rehydrated with 10 ml. of a suitable concentration of sodium chloride to give an isotonic concentration of salts in the final suspension and an activity of 29 L_f/ml.

All preparations, contained in ampoules, were frozen in an alcohol bath at $-45°$ C before drying. Following freezing the material was dried by sublimation *in vacuo* in a chamber freeze-dryer (Usifroid, Model SMU) to a residual moisture content of less than 1 % and sealed under dry nitrogen (Rey, 1960; Rieuford, 1960).

In those preparations of diphtheria toxoid plus tetanus toxoid alone or in combination with pertussis vaccine, dried by sublimation *in vacuo* and sealed under air, the concentration of the starting material was adjusted so that following rehydration each ml. of the former contained 21 L_f units and each ml. of the latter 35 L_f units.

The activity of dried toxoid was determined before exposure to elevated temperatures and after exposure to (1) 100° C. for 60, 80, 100, 120 and 140 min., (2) 110° C. or 120° C. for 60 min., (3) 130° C. for 60, 120 or 180 min.

RESULTS

The effects of exposure to elevated temperatures for 60 min. on the L_f or K_f values of dried diphtheria toxoid alone and in combination with tetanus toxoid or tetanus toxoid plus pertussis vaccine are shown in Table 1. The L_f values for all preparations sealed under nitrogen, exposed to elevated temperatures and tested immediately thereafter did not vary significantly from those of the control. Significant decreases in L_f values were observed in those samples sealed under air. The greatest change occurred in dried diphtheria toxoid in combination with tetanus toxoid when heated to 130° C. for 60 min. (57 %). The L_f values of the foregoing preparations did not change following storage at room temperature (20° C.) for 2 years. The decline in the diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine was 17 % when heated to 130° C. for 60 min.

K_f values increased with each increment of increased temperature in preparations of dried diphtheria toxoid only (nitrogen), dried diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine (nitrogen) or dried diphtheria toxoid when in combination with tetanus toxoid plus pertussis vaccine (air). Results similar to the above were observed for dried diphtheria toxoid plus tetanus toxoid (nitrogen) heated to a final temperature of 120° C.; the K_f value of 130° C. was obtained following the addition of untreated diphtheria toxin and consequently the time required for flocculation was shortened.

In the samples stored at room temperature for 2 years and tested with the addition of untreated diphtheria toxin, the time for flocculation increased significantly following exposure at 100° C. or 110° C. and declined significantly following exposure at 120° C. or 130° C.

In experiments similar to the above, the L_f values for samples sealed under nitrogen did not change following heating to 100° C. for 80, 100, 120 or 140 min.; significant decreases were observed in samples sealed under air. The L_f values for samples of diphtheria toxoid only and diphtheria toxoid when in combination

with tetanus toxoid, both sealed under nitrogen, did not change following heating to 130° C. for 120 and 180 min.; flocculation was aided by the addition of untreated diphtheria toxin.

The K_f values of the preparation above increased with prolonged exposure to

Table 1. *The effects of exposure to elevated temperatures for* 60 *min. on the* L_f *and* K_f *values of diphtheria toxoid alone and in combination with tetanus toxoid or tetanus toxoid plus pertussis vaccine dried by sublimation* in vacuo *and sealed under dry nitrogen or air*

	L_f/ml.					K_f in min.				
Temperature (°C)	Control (dried and rehydrated only)	100°	110°	120°	130°	Control* (dried and rehydrated only)	100°	110°	120°	130°
Diphtheria toxoid (nitrogen)	35	35	35	35	35	80	90	200	255	720
Diphtheria toxoid + tetanus toxoid (nitrogen)	35	35	35	35	35	110	165	370	595	320‡
Diphtheria toxoid + tetanus toxoid†‡ (air)	21	21	15	13	9	165	250	380	210	210
Diphtheria toxoid + tetanus toxoid + pertussis vaccine (nitrogen)	29	29	28	27	27	125	145	205	300	490
Diphtheria toxoid + tetanus toxoid + pertussis vaccine (air)	35	34	31	30	29	100	120	175	275	480

* The K_f values of the original fluid preparations before freezing and freeze-drying were approximately 30 minutes.

† These samples were stored for two years at room temperature.

‡ Flocculation aided by the addition of active diphtheria toxin.

Table 2. *The effects of the delayed additions of serum (antitoxin) on the* L_f *and the* K_f *values of diphtheria toxoid dried by sublimation* in vacuo *and exposed to* 100° *C. for* 60 *min. before rehydration and testing*

	Flocculation at 20° C				Flocculation at 45° C			
	Antitoxin added immediately		Antitoxin delayed		Antitoxin added immediately		Antitoxin delayed	
Antigen tested	L_f/ml.	K_f in min.	L_f/ml.	K_f in min.	L_f/ml.	K_f in min.	L_f/ml.	K_f in min.
Dried diphtheria toxoid before heating (control)	30	342	30	365	30	91	29	141
Dried diphtheria toxoid following heating at 100° C. for 60 min,	30	357	29	345	30	168	30	177

100° C. The values for K_f of preparations heated to 130° C. for 120 min. declined following the addition of untreated diphtheria toxin; an increase in K_f was observed in preparations exposed for 180 min.

In other studies, constant amounts of antigen (diphtheria toxoid dried by sublimation *in vacuo*, exposed to 100° C. for 60 min. and rehydrated with 10 ml. of a concentration of NaCl sufficient to result in an isotonic solution) were placed in two sets of tubes and varying amounts of serum were added immediately to one set. Several tubes of both sets were kept at 20° C. (room temperature) or at 45° C. When flocculation appeared in the set of tubes containing the antigen-antibody complex, varying amounts of serum were added to the tubes containing antigen only.

The variations in the values of L_f for the above were not significant (Table 2). The variations in the values of K_f between sets of tubes containing diphtheria toxoid treated similarly and differing only in the time at which serum was added and the temperature at which the test was carried out, with one exception, were not significant.

DISCUSSION

The flocculation reaction is based primarily on the specific, mutual attraction of antigen and antibody. Although the reaction is usually completed in 1–2 min., in some cases several hours may elapse. In our studies the latter occurred when a weakly combining diphtheria toxoid antigen was used. To increase the sensitivity of the test system an active diphtheria toxin of known L_f value was added to the weakly reacting system. Through the use of 'helper' toxin we were able to demonstrate residual activity in preparation of diphtheria toxoid dried by sublimation *in vacuo* and stored under adverse conditions (sealed under air) or exposed to high temperatures. The need for 'helper' toxin may indicate that various degrees of denaturation of antigens exist. If increased K_f values are a sign of slight damage, the necessity for the addition of an active antigen, diphtheria toxin, to obtain flocculation is probably a sign of greater damage. The observation that original L_f values can be obtained in preparations stored under adverse conditions (dried diphtheria toxoid when in combination with tetanus toxoid, sealed under air and stored two years) supports the above.

In the preparations dried by sublimation *in vacuo* and sealed under nitrogen, heating did not alter L_f values; K_f values were increased. These data indicate that heating did not affect the total amount of diphtheria toxoid protein available for flocculation, but resulted in some denaturation of protein components responsible for their specific attraction to antibody. The finding that slight alterations in the structure of a hapten caused considerable changes in the affinity of hapten for antibody lend support to the above (Haurowitz, 1963). These results were similar to those obtained when dried antibody was exposed to elevated temperatures following freeze-drying (Damjanovic & Iovicic, 1964). It is to be noted also that freezing and drying by sublimation *in vacuo* of itself resulted in denaturation as shown by the increased K_f values for non-heated, dried preparations.

If we assume that the principal factor involved in the heating of dried diphtheria toxoid was reversible changes in protein structures of the antigen responsible, in

part, for the affinity of antigen for antibody, one would expect the K_f's of heated dried diphtheria toxoid to give normal values if rehydrated and stored before the addition of serum. The results reported by Holt (1950), decreased K_f on prolonged storage at room temperature, support this hypothesis. That the above did not occur in our system is shown by the results obtained when the addition of the serum was delayed (Table 2). The differences observed in the direction of K_f values may be a function of the temperatures and the periods of storage.

Accelerated deleterious changes in the stabilities of biologic materials have been reported for dried suspensions sealed under air in contrast to those sealed under vacuum or dried nitrogen (Sololov, Kulikova, Kholeva & Azadova, 1958). In our studies decreased L_f and increased K_f values were observed in dried toxoid sealed under air and exposed to elevated temperatures. The decline in activity of the former occurred as a first-order reaction. It is recommended, therefore, that dried diphtheria toxoid be stored under nitrogen rather than air for increased stability.

The combination of diphtheria toxoid with tetanus toxoid or with tetanus toxoid plus pertussis vaccine does not seem to have any significant influence on the stabilities of dried diphtheria toxoid. The difference in K_f values between diphtheria toxoid only and diphtheria toxoid when in combination with tetanus toxoid could be anticipated; the flocculation of the latter is usually slower (Regamey, 1957). The slight decrease in L_f values when diphtheria toxoid is in combination with tetanus toxoid plus pertussis vaccine (nitrogen) does not seem sufficient reason to assert that it was caused by the presence of pertussis vaccine.

SUMMARY

The L_f values of diphtheria toxoid alone and in combination with tetanus toxoid or tetanus toxoid plus pertussis vaccine, dried by sublimation *in vacuo*, sealed under nitrogen, exposed to elevated temperature and rehydrated thereafter were not altered. L_f values declined in samples sealed under air. The values for K_f in the above preparations increased in relation to increased temperatures of exposure for a given time or following exposure to a given temperature for increased time intervals. The sensitivity of the system of testing used was greater following the addition of 'helper', a fast flocculating solution of diphtheria toxin, and in the case of dried diphtheria toxoid stored under adverse conditions (sealed under air) for two years, the addition of 'helper' was necessary to obtain a flocculation reaction. In general, the results obtained indicated a very high stability for preparations sealed under nitrogen, and a significantly lower stability for preparations sealed under air.

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The effect of sex and age on the response to warfarin in a non-inbred strain of mice*

By F. P. ROWE and R. REDFERN

Infestation Control Laboratory, Ministry of Agriculture, Fisheries and Food, Tolworth, Surbiton, Surrey

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INTRODUCTION

The results of a study of the toxicity of warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] to wild house-mice (*Mus musculus* L.) indicated that there was a difference in susceptibility attributable to sex (Rowe & Redfern, 1964). In free-feeding tests lasting from 4 to 28 days, proportionally more male than female mice were killed after feeding on bait containing 0·025 % warfarin and the average time to death of females was longer than that of males. Furthermore, although the mice tested were of unknown age, the data also suggested that the heaviest, and therefore probably the oldest females were the most difficult animals to kill. These findings prompted the following further investigation of the possible influence of sex and age on the response to warfarin in mice.

METHODS

The mice employed in this study were a non-inbred strain (L. A. C. Grey) developed at the Laboratory Animals Centre, Medical Research Council Laboratories, Carshalton. Preliminary sighting tests showed that this laboratory strain of mice was more susceptible to warfarin poisoning than the wild species. Twelve out of twenty L.A.C. Greys were killed after feeding on bait containing 0·005 % warfarin for 3 days compared with only six out of thirty wild mice fed on bait containing 0·025 % warfarin over the same period.

One hundred (fifty male and fifty female) L. A. C. Grey mice were paired at random when they were 6 weeks old; each pair was placed in a container measuring 12 × 5 × 4 in. and supplied with a composite diet (diet 41*b*) and water *ad lib.* The date of birth of litters was recorded and the litters taken from their parents when they were 3 weeks old. Littermates were housed together until they were 5 weeks of age and then isolated in metal test-cages measuring 14 × 11 × 6 in.

The adult pairs were allowed to breed a second time and each male was removed from its breeding female as soon as the latter was again clearly pregnant. The dates of birth of second litters were recorded and the young removed from their mothers when they were 3–4 weeks old. Littermates of the same sex were kept together until they had reached the age of 22 weeks at which time they were isolated in test cages. Parent mice were similarly caged 2 weeks prior to poisoning.

Apart from some of the females of second litters, all mice were tested in the same manner. Each animal was weighed and on the following day diet 41*b* was removed from its cage; it was then presented with a plain bait-base consisting of pinhead oatmeal, caster sugar, and a technical grade 'white oil' in the proportions by weight of 17:1:1 respectively for 4 days. At the end of this period an excess amount of bait-base containing 0·005 % warfarin was offered to the mouse daily for 3 days. The poison was dispersed thoroughly in fine oatmeal to give a master-mix containing 0·1 % warfarin and one part of the master-mix was then added to 19 parts of the bait-base. The total amounts of bait-base and poison bait eaten were recorded, paper placed in the tray underneath each cage facilitating the collection of spilled food. After the end of the 3-day test period, the warfarin bait was replaced by diet 41*b*. The times of death and the weights of mice that died were recorded and the dead animals autopsied. Animals tested in this manner included the established breeding pairs when they were 6 months old, first litter males and females at the age of 6 weeks, and all the males and a proportion of the females of second litters at the age of 6 months.

The remaining females from second litters were used in two toxicity tests undertaken to determine the effect on the mortality level of incorporating the substituted male hormone, methyl testosterone, in their feed. In both these tests, animals were divided into two groups so that as far as possible each group contained the same number from a given litter. In the first test, nine females were offered bait containing 0·005 % warfarin, and ten others bait containing 0·05 % methyl testosterone and 0·005 % warfarin, each over a period of 3 days. In a later test, thirty females were fed on crushed diet 41*b* containing 0·05 % methyl testosterone for a 2-week period before they were presented with bait containing 0·005 % warfarin and 0·05 % methyl testosterone for 3 days; simultaneously a control group of twenty-nine females were offered bait containing only 0·005 % warfarin for 3 days. Details of other tests are given below.

RESULTS

The results of the 3-day free-feeding toxicity tests with bait containing 0·005 % warfarin are summarized in Table 1.

The data in Table 1 show that there was a clear-cut sex difference in response to warfarin poisoning in mice 6 months old, significantly more male than female parent and second litter mice succumbing to the toxic effects of warfarin. Although the mean body weight of male animals was greater than that of females the higher comparable mortality of 6 month old males cannot be attributed to any greater food intake on their part: for adult female mice survived better than adult males despite consuming on average a higher dose of poison (Table 2). No sex difference in the action of warfarin was evident in mice 6 weeks old and there was no marked difference in the amount of poison consumed by surviving males and females of this age class.

The results of the two experiments in which adult female mice were administered methyl testosterone in their diet are given in Table 3.

No marked differential mortality occurred between adult female mice treated with either warfarin bait alone or with bait containing warfarin and methyl testosterone over a 3-day period. The average dose of warfarin received by mice in each of the two groups during this period was 23·2 and 20·0 mg./kg. respectively.

Table 1. *Effect of sex and age on toxicity of warfarin in L.A.C. Grey mice.*

Group	Age	Mortality	Mortality (%)	Significance of difference
Parent males	6 months	35/46	76·1	$P = < 0{\cdot}001$
Parent females	6 months	7/47	14·9	
First litter males	6 weeks	44/141	31·2	$P = 0{\cdot}1$–$0{\cdot}5$
First litter females	6 weeks	37/138	26·8	
Second litter males	6 months	80/120	66·7	$P = < 0{\cdot}001$
Second litter females	6 months	14/44	31·8	

Table 2. *The average dose of warfarin consumed by male and female L.A.C. Grey mice of two age classes*

Sex	Result	Number	Average weight (g.)	Average total warfarin dose (mg./kg.)
Parents (6 months)				
M	Died	35	37·5	13·4
	Survived	11	39·3	16·3
F	Died	7	32·1	15·2
	Survived	40	33·0	21·6
First Litters (6 weeks)				
M	Died	44	21·1	22·5
	Survived	97	20·0	27·4
F	Died	37	18·2	26·2
	Survived	101	18·2	25·8
Second Litters (6 months)				
M	Died	80	38·1	15·7
	Survived	40	38·8	20·3
F	Died	14	28·5	13·7
	Survived	30	31·0	26·6

Table 3. *Effect of methyl testosterone on mortality in 6-month-old female mice fed on bait containing* 0·005 % *warfarin*

Treatment	Days	Mortality	Mortality (%)
0·005 % warfarin	3	1/9	11·1
0·005 % warfarin plus 0·05 % methyl testosterone	3	0/10	0·0
0·005 % warfarin	3	9/29	31·1
0·005 % warfarin plus 0·05 % methyl testosterone*	3	16/30	53·3

* Having first been fed for 2 weeks on bait containing 0·05 % methyl testosterone

The response to warfarin in the second test was greatest in those females presented with methyl testosterone in their feed for 2 weeks before they were poisoned with warfarin, but the difference in mortality between treated and untreated animals was not significant ($\chi^2 = 2{\cdot}2$, $P = 0{\cdot}1$–$0{\cdot}5$). It is possible, however, that the mortality in females pre-treated with methyl testosterone would have been greater had they eaten as much as the untreated females. The pre-treated mice ate 15·2 mg./kg. on the average and the untreated females ate 19·5 mg./kg.

In a similar test undertaken later, female littermates were divided into three groups soon after they were weaned. When they were 6 months old mice in each of the three groups were offered 0·005% warfarin bait over a 3-day period. Females of the first group were given methyl testosterone in their feed for 2 weeks before poisoning; the second and third groups of females were subjected to warfarin poisoning only, animals in the last group, however, being first allowed to breed. The mortality obtained in each of the three groups of females was 10/25, 7/26 and 8/26 respectively.

DISCUSSION

The response of laboratory rats and mice to a number of chemical substances has been observed to vary with sex. For example, Quinn, Axelrod & Brodie (1958) showed that the duration of action of hexobarbitone was about four times longer in female rats than in males, while DuBois, Doull, Salerno & Coon (1949) found that parathion was more toxic to female than to male rats.

Venho (1959) studied the effect of sex on the toxicity of the anticoagulants dicoumarol and phenylindanedione to mice and found that females were more resistant than males to these drugs. A similar sex variation in response to warfarin was found in adult laboratory mice in the present study and supports the earlier observation made on wild mice (Rowe & Redfern, 1964).

Warfarin is antagonistic to vitamin K and the symptoms of warfarin poisoning and vitamin K deficiency—hypoprothrombinemia and haemorrhage—are identical. Metta & Johnson (1960) who maintained rats on a diet deficient in vitamin K found that females were less susceptible than males to vitamin K deficiency. It is considered probable therefore that female mice survived warfarin poisoning better than males in the present study because, like female rats, they are less susceptible to vitamin K deficiency.

Quinn *et al.* (1958) further showed that the metabolism of hexobarbitone could be influenced by sex hormones, oestradiol administration increasing the response of male rats to the drug and testosterone reducing the response of females. From the results of their study Metta & Johnson (1960) concluded that the greater susceptibility of male rats to an artificially induced vitamin K deficiency was not associated with coprophagy or due to any higher food intake on their part compared with females but was a true sex difference under hormonal control. Some evidence that sex hormones also play a part in determining the response of male mice to anticoagulants was provided by Venho (1959) who found that the administration of oestradiol to castrated male mice decreased the mortality due to dicoumarol. Further indirect evidence to support the viewpoint that sex hormones influence

the response of male mice to anticoagulants is given by the mortality data (Tables 1 and 2) of immature and mature male animals given warfarin. A marked lower mortality occurred in the younger animals although on average they ate relatively more poison than the older animals—showing that male mice become more susceptible to warfarin poisoning with maturity. This result is in accordance with the work of Mellette & Leone (1960) who kept male rats of differing ages on a diet deficient in vitamin K and found that susceptibility to hypoprothrombinemia increased with age.

These authors also found the reverse situation in female rats, the latter becoming less susceptible to a lack of dietary vitamin K with age. Furthermore, they reported that the decline in the prothrombin level of female rats maintained on a vitamin K free diet and injected with testosterone propionate was greater than that of females maintained on a vitamin K free diet only. There was no evidence in the present study, however, that female mice become more resistant to warfarin with age. The mortality in female mice 6 weeks old was not significantly different from that of either 6-month-old parent or second litter females ($\chi^2 = 2{\cdot}1$, $P = 0{\cdot}1$–$0{\cdot}5$; $\chi^2 = 0{\cdot}2$, $P = 0{\cdot}5$–$0{\cdot}9$ respectively), despite the fact that again the younger animals consumed relatively more poison (Tables 1 and 2). Although the mortality data of Table 1 suggested the possibility that 6-month-old females that had bred were more susceptible to warfarin than virgin animals of the same age, this was not substantiated by further experiments. The present experiments also showed that the prior administration of methyl testosterone did not significantly enhance the toxicity of warfarin to adult female mice and this is similar to the finding of Venho (1959) that the administration of testosterone to castrated female mice did not change the mortality due to dicoumarol.

SUMMARY

1. A marked sex variation in susceptibility to warfarin was found in 6-month old L.A.C. Grey mice, males being more susceptible than females. No sex difference in response was evident in young mice 6 weeks old.

2. Susceptibility to warfarin poisoning increased markedly with age in males but not in females.

3. No significant increase in mortality following warfarin poisoning occurred in adult female mice given methyl testosterone.

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Experimental infection of Common Terns with Tern virus: Influenza Virus A/Tern/South Africa/1961

By W. B. BECKER

C.S.I.R. and U.C.T. Virus Research Unit,
University of Cape Town, South Africa

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In 1961 an epizootic occurred among Common Terns along the coastline of the Cape Province, South Africa, and the causative virus was isolated and classified as Influenza A/Tern/South Africa/1961 (Becker, 1966). The pathogenesis of experimental Tern virus infection has been described in chickens (Becker & Uys, 1967; Uys & Becker, 1967), but a method of capturing live, healthy terns was eventually devised and fifteen Common and two Swift Terns were obtained for experimental purposes. The aims with the Common Terns were to confirm that the disease seen during the epizootic could be reproduced experimentally, to study the distribution of virus in the tissues of infected birds, and to determine possible means of spread of infection in the field. The Swift Terns were obtained with a view to confirming earlier observations that they suffered no ill-effects from the injection of Tern virus.

MATERIALS AND METHODS

Virus strains

The prototype strain of Tern virus (Becker, 1966) was used at the first or second allantoic passage in embryonated eggs.

Chickens and eggs

Leghorn-Australorpe-cross chickens and eggs were used.

Terns

The Common Terns (*Sterna hirundo*) and the Swift Tern (*Sterna bergii*), like the chickens, were housed in 24 in. × 18 in. × 12 in. cages. The terns were kept healthy and adequately nourished, but the Common Terns required force-feeding; the diet consisted only of sea-fish given two or three times per day.

Autopsy, virus titration, haemagglutination-inhibition (HI) tests

The details of these procedures have been described previously (Becker, 1966).

Immunofluorescence

Immunofluorescent studies were performed on tissues which were removed at autopsy, fixed in cold ethanol and embedded in low-melting-point paraffin wax according to the method of Sainte-Marie (1962). Sections were stained for viral

Table 1. *Virological data on experimentally infected Common Terns*

Tern no.	Inoculation with Tern virus: EID_{50}*	route	Result	Route of re-inoculation with 10^4 EID_{50} of Tern virus	Result	Post mortem virus titrations: Blood	Muscle	Liver	Lung	Heart	Kidney	Brain	Cloacal swab	Palatal swab
1	10^4	CN	Died within 3 days	—	—	6·9†	6·5	5·3	6·5	5·5	6·7	3·3	+	+
2	10^4	IM				5·7	4·7	5·3	6·5	5·9	6·9	3·5	NE	NE
3	10^4	IM				1·7	3·7	3·9	5·5	5·9	ND	3·9	3·0	3·0
4	10^3	CN	Unaffected	IM	Died < 3 days	NE	> 4·5	5·9	5·9	6·9	5·9	4·3	3·5	3·0
5	10^2	CN		IM	Died < 3 days	6·3	4·7	4·1	6·5	5·1	6·5	2·9	> 3·5	4·1
6	10	CN		CN	Died < 4 days	4·3	4·7	4·3	6·0	4·9	6·7	4·3	4·1	4·1
7	10	CN		CN	Recovered. Autopsied on 14th day. HI antibody titre > 40	NE	—	—	—	—	—	—	—	—
8	1	CN		IM	Died < 2 days	5·5	4·9	4·5	5·9	4·5	4·1	3·1	NE	—
9	1	CN		CN	Died < 7 days	3·9	4·1	—	5·9	3·9	5·1	4·1	—	3·3

* Egg infective $doses_{50}$.
† Log_{10} EID_{50} per g.
CN, Conjunctival/intranasal; +, virus present; NE, not examined; IM, intramuscular; —, no virus present.

antigen by the indirect method using Tern virus strain-specific antiserum prepared in guinea-pigs, followed by a rabbit anti-guinea-pig gamma globulin labelled with fluorescein-isothiocyanate (Antibodies Incorporated, Davis, California).

RESULTS

Controls

Three Common Terns and one Swift Tern were used as uninfected controls and autopsied on receipt at the laboratory; no abnormalities were detected and no histological or virological evidence of infection with Tern virus was obtained. A fourth Common Tern died on the fifteenth day of captivity owing to difficulty with feeding, and a fifth died from pulmonary aspergillosis (*Aspergillus fumigatus*) after 3 weeks in captivity; these also showed no evidence of Tern virus infection.

Experiment 1

Approximately 10^4 egg infective doses $(EID)_{50}$ of freshly passed Tern virus were administered to one Common Tern by the conjunctival/intranasal (CN) route; half the inoculum was introduced into the right conjunctival sac and half into the right nostril. Two Common Terns were injected intramuscularly (IM) with the same amount of virus. All three birds died within 3 days of infection during an illness which showed the following features: the birds became apathetic and their feathers were ruffled; then their heads and wings drooped and they preferred to close their eyes; soon they were no longer able to remain standing but gradually sagged to the ground and finally collapsed and died (Plate 1). Virus was found in all the specimens taken at autopsy including blood, breast muscle, liver, lung, heart, kidney, brain, cloacal and palatal swabs (Birds 1, 2, 3, Table 1). The droppings of one bird which was injected IM were examined twice daily for Tern virus, which was detected on the day preceding death. No HI antibodies to Tern virus were detected in pre-inoculation serum samples tested at an initial dilution of 1/5. Control 3-day-old chickens received the above dose of Tern virus CN or IM and all died showing the features of Tern virus infection previously described (Becker & Uys, 1967).

Experiment 2

Six Common Terns (birds 4–9, Table 1) received from 1 to 10^3 EID_{50} of Tern virus by the CN route, but the terns were unaffected by these doses of virus. No HI antibodies were found in pre-inoculation serum diluted 1/5, and 9 days after inoculation titres of 5 were found only in birds 7 and 9. The Swift Tern was injected IM with 10^3 EID_{50} of Tern virus and had developed an HI antibody titre of 2560 nine days later without ill-effect. Half of the control 3-day-old chickens died after inoculation CN with 10^3 EID_{50} of Tern virus.

Experiment 3

The six Common Terns from the previous experiment were re-inoculated after an interval of 19 days with 10^4 EID_{50} of Tern virus using the CN or IM route (birds 4–9, Table 1). The three terns injected IM died within 3 days; one inoculated

by the CN route died within 5 days, another within 7 days, and the third became ill but recovered and had a serum HI antibody titre of over 40 when it was autopsied on the 14th day. Virus was detected in the autopsy specimens from all except the last-mentioned bird. The Swift Tern was re-injected using 10^4 EID_{50} of Tern virus: it showed no ill effect, maintained its HI antibody titre and no virus was isolated from its tissues at autopsy 2 weeks later. Four 3-week-old chickens inoculated CN and five 3-day-old chickens injected IM served as controls; all died within 7 days.

Experiment 4

Lice (*Austromenopon* species) were transferred daily from the Common Terns used in Exp. 3 to another Common Tern which remained well during the observation period of 7 weeks: no virus was isolated from its tissues. No virus was cultured from eight lice taken from Tern 4 (Table 1) at the time of autopsy. The diet of these lice consists principally of dead feather material.

Immunofluorescence

Immunofluorescent studies were carried out on all the Common Terns. Tern virus replication was only demonstrable in the tissues of the eight fatally infected terns. The sites of virus replication were in the lung in all eight birds, in the heart, skeletal muscle and brain in seven, in the kidney in three, in the spleen in all five cases in which it was examined, and in the cloacal and palatal glands in two of six birds. No specific fluorescence was detected in the liver, and the blood clot and blood smears of two terns were also examined with negative results.

CONCLUSIONS

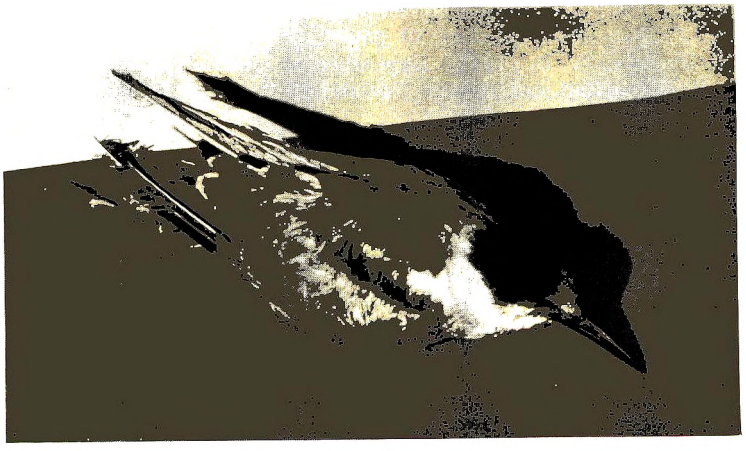
The acute illness with high mortality which affected Common Terns in the 1961 epizootic was reproduced experimentally in the original host species. The same results were obtained by either CN or IM routes of infection, but a dose of 10^4 EID_{50} was necessary to produce fatal infection by CN inoculation. At death the Common Terns had a viraemia and consequently virus was detected in all the tissues; however, immunofluorescent studies showed that the sites of virus replication were usually in muscle, heart, brain, lung, spleen, and sometimes kidney, but not in the liver. Virus was usually isolated from the palatal and cloacal swabs of the birds and viral antigen was demonstrated in the glands of the palate and cloacal mucous membrane in some birds by means of specific immunofluorescence. It was confirmed that a single dose of Tern virus injected IM had no apparent ill effect on the Swift Tern, but produced high titre circulating antibodies.

DISCUSSION

Cloacal and palatal secretions or excretions seem the likely source of virus for spread of infection, which might occur from contact with excreta, or as a result of fighting or sexual activity. In the 1961 epizootic the preceding conditions of stress might have converted a latent into an overt infection and precipitated the outbreak by lowering the threshold of resistance and thus favouring the spread

(*a*)

(*b*)



(*c*)

of the infection. The role of blood-sucking arthropods in the spread of this infection is unknown. Sea-birds may be a source of infection of domestic poultry with myxoviruses, as discussed in a previous paper (Becker, 1966). The outbreak in chickens in Scotland in 1959 (Dr J. E. Wilson, personal communication) and the Tern epizootic in 1961 were caused by influenza A viruses with closely related strain specific antigens which were unrelated to those of any previously known influenza A viruses. Recently strains of influenza A related to the Tern and Scottish viruses were isolated from turkeys in Canada (Dr G. Lang, personal communication). This lends further support to the hypothesis that migrating sea-birds such as the Common Tern may transmit avian influenza A viruses to domestic poultry.

SUMMARY

Experimental infection of captive Common Terns with Influenza virus A/Tern/South Africa/1961 reproduced the disease seen in the 1961 epizootic during which Tern virus was originally isolated. Infected terns excreted virus in their droppings. At death a viraemia was present but immunofluorescent studies determined the sites at which virus reproduction occurred. A Swift Tern suffered no ill effect from the injection of Tern virus but developed HI antibodies. The role of migrant sea-birds in spreading avian influenza is briefly discussed.

The capture of the terns by Mr L. M. J. Keyzer is gratefully acknowledged. Thanks are due to Prof. A. Kipps, to Prof. C. J. Uys for the photographs, and to Misses E. Baker and K. Larsson for technical assistance.

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EXPLANATION OF PLATE

Common Terns, approximately one quarter life size.

(*a*) Healthy Common Tern (No. 2, Table 1). Photograph taken before inoculation with Tern virus.

(*b*) and (*c*) Sick Common Tern (no. 3, Table 1) photographed in the late stages of infection at 68 and 70 hr. respectively after inoculation with Tern virus. The bird died at 71 hr.

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Genetic marker studies of poliovirus

I. Natural variation

By YVONNE E. COSSART
Virus Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

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Intratypic variation of poliovirus was first recognized by Ramos-Alvarez & Sabin (1954) when they isolated from the faeces of healthy children naturally attenuated strains of poliomyelitis types 2 and 3 which were avirulent for monkeys by intracerebral inoculation. Antigenic variation, extensively studied by Wenner, Archetti & Dubes (1959) occurs in each serotype, but all strains of a single type share major antigens to such a large extent that special methods such as the kinetic neutralization test (McBride, 1959) or plaque inhibition (Wecker, 1960) are required to demonstrate the differences.

Dubes & Chapin (1956) were able to select variants of type 1 which replicated almost as well at 30° as at 36° C. and suggested that this alteration was accompanied by attenuation of the strains. This was applied (Sabin, Hennessen & Winsser, 1954) in the artificial production of attenuated strains for use in poliovirus vaccines. Lwoff (1962) developed the 'reproductive capacity temperature' (R.C.T.) marker test and pointed out that the naturally virulent prototype strains were able to grow as well at the 'fever temperature' of 40° as at 37° C., but were almost completely inhibited at 30° C. The attenuated vaccine strains behaved in converse fashion. He suggested that this ability to grow at 'fever' temperature is causally related to virulence.

Many other genetic marker tests have since been described. Some depend on the tissue culture host range (Kanda & Melnick, 1959; Kelly, 1962); some on resistance to physical agents such as heat (Stanley, Dorman, Ponsford & Larkin, 1956); others on growth requirements, e.g. for cystine (Dubes & Chapin, 1958); while an interesting group (Bengtssen, Philipson, Persson & Laurent, 1964) measures adsorption of the infective particles to charged molecules such as aluminium salts (Wallis, Melnick, Terry & Wimberley, 1962) or dextran sulphate (Takemoto & Kirschstein, 1964).

These tests have mostly been applied to prototype strains, including both naturally derived and vaccine strains, and relate especially to type 1. Their immediate practical value is to act as *in vitro* indicators of the relative virulence of different strains and to detect the possible derivation of strains from live attenuated vaccine.

Tests designed to measure virulence are usually compared with the monkey neurovirulence test as standard (Sabin & Lwoff, 1959). It has been shown (Benyish-Melnick & Melnick, 1959) that there is good correlation between the R.C.T. test

and monkey neurovirulence for prototype strains, but vaccine trials (Koprowski *et al.* 1960; Magrath, Boulgar & Hartley, 1964) have revealed a tendency for this laboratory character of the attenuated strains to revert towards that of the wild strains after quite short periods of intestinal passage in man. This finding has given impetus to the study of properties which do not vary directly with virulence, and so may be more useful as genetic markers in epidemiological studies.

Although there has been much work on vaccine progeny, little information is available about the natural variation of polioviruses with respect to these 'marker' properties. It was therefore decided to examine strains collected in this laboratory before the introduction of vaccination by three tests: (*a*) the R.C.T.$_{40}$ test (Lwoff, 1962), (*b*) serodifferentiation by a modification of the Wecker technique (Nakano & Gelfand, 1962), and (*c*) (for type 1 strains only) inhibition of growth by dextran sulphate (Cossart, 1966). The R.C.T.$_{40}$ and serological tests were chosen because they have been applied extensively in other laboratories, while the dextran sulphate inhibition was included as an economical method of measuring an adsorption property.

MATERIALS AND METHODS

Strains

The viruses included in the study may be divided into the following groups:

Type 1. (*a*) 1953: obtained from clinical cases of poliomyelitis which occurred in different areas of the United Kingdom (Goffe, 1955)—21 strains. (*b*) 1957: isolated from clinical cases of poliomyelitis in that year—34 strains.

Type 2. 1953: obtained from cases of poliomyelitis in the epidemic of that year—18 strains.

Type 3. 1953–54. Only a few strains were available, reflecting the sporadic occurrence of type 3—5 strains.

The isolations had been carried out in primary monkey kidney cell culture and strains were then stored at $-30°$ C. The earliest pass available was used provided its titre was $10^{5\cdot5}$ TCD50/0·1 ml. or more. The passage numbers and clinical diagnosis are listed in the tables.

The prototype strains are listed below:

	Naturally occurring	Vaccine
Type 1	Mahoney	Sabin 1
Type 2	YSK	Sabin 2
Type 3	Saukett	Sabin 3

The naturally occurring strains have been passaged in monkey kidney cells in this laboratory since they were obtained as monkey testicular passes from Dr J. E. Salk.

The vaccine strains were obtained from Dr A. B. Sabin and used as the fourth monkey kidney passage in this laboratory.

Tissue culture

Primary rhesus monkey kidney cell cultures were used for tube titration. Primary or secondary rhesus monkey kidney or primary patas monkey kidney cells were used as monolayers in Petri dishes for plaque assays.

The R.C.T.$_{40}$ *marker test*

This was performed by titrating each strain in parallel at 36° C. and at a higher temperature which differed according to the serotype, 39·8 ± 0·2° C. for types 1 and 2 and 40·3 ± 0·2° C. for type 3. This upper temperature of 39·8° C. was chosen as a compromise between the classical 40° and 39·5° which is recommended by the Biological Standards Control Division, Medical Research Council, who also choose 40·3° C. for type 3 (D. I. Magrath, personal communication). These temperatures are the critical ones at which the Sabin strains are only just completely suppressed while at the same time the wild prototype strains show very slight reduction in titre. The titrations at the higher temperatures were incubated in a well-insulated incubator with a fan to maintain air circulation. Tests with a thermocouple and recording device showed the temperature was maintained within the stated range of ± 0·2° C.

The virus suspensions were titrated in $\log_{10}$ steps and four monkey kidney culture tubes were inoculated with 0·1 ml. of each dilution. Synthetic medium 199 was used for maintenance. Two tubes per dilution were incubated at 36° C. and two at the higher temperature. Cytopathic effects were observed on the 3rd and 6th day and the end-point calculated from the 6th-day reading. Naturally derived and vaccine prototype strains of the appropriate serotype were included in each test. A satisfactory test showed less than 2 $\log_{10}$ TCD 50 reduction in titre of the wild strain while the titre of the vaccine strain was reduced by 5 $\log_{10}$ TCD 50 or more at the higher temperature (Sabin, 1961).

The serological marker test

This was performed by a modification of the Wecker technique (Nakano & Gelfand, 1962).

Preparation of sera. At weekly intervals rabbits were given intravenous injections of monkey kidney tissue culture fluid containing approximately 10^8 TCD 50 of prototype virus in 5 ml. They were bled from the ear 10 days after the last injection. A number of antisera were made against each prototype vaccine and wild strain and those which showed a fourfold or greater difference in titre in standard tube neutralization tests between the vaccine and wild strains of the same serotype were selected for use. These sera were then titrated by the Wecker method and the concentration of serum which was just sufficient to give 95 % plaque reduction of the homologous strain was used in subsequent tests.

Method of the test. Monolayers of monkey kidney cells were grown in 60 mm. plastic Petri dishes (Sterilin). For each strain the medium was poured from six dishes and 0·2 ml. of the virus suspension containing about 30 TCD 50 was inoculated into the centre of each dish. Virus was allowed to adsorb during 90 min.

at 37° C. in the CO_2 incubator and dishes were then overlaid as follows: Two dishes with 'plain' agar (1·5 % Bacto Difco agar in Medium 199), two dishes with agar containing the appropriate dilution of antiserum to the wild prototype and two dishes with agar containing antiserum to the vaccine prototype strain of the same serotype. When the agar had set the dishes were inverted and returned to the CO_2 incubator for 2 days. They were then overlaid a second time with agar containing 1/10,000 neutral red (Vital fluorochrome, Gurr). After overnight incubation plaques were counted and the percentage reduction under each serum was calculated.

So that strains could be compared, an index was derived from these values, thus:

$$\text{serological index} = \frac{\%\text{ plaque reduction under anti-Sabin-strain serum}}{\%\text{ plaque reduction under anti-wild-strain serum}}.$$

For type 3, however, a strain-specific anti-Saukett serum was not obtained despite repeated attempts, and the results for type 3 strains are given as percentage plaque reduction under anti-Sabin 3 serum. With each test wild and vaccine prototype strains of the same serotype were included, but no correction has been made for variation in the serological index of the control strains.

Table 1. *Results of marker tests on prototype strains given as the average of five determinations with the range of values shown in brackets*

Strain	R.C.T.$_{40}$ ($\log_{10}$ difference)*	Serological index†	Dextran marker ($\log_{10}$ difference)
Sabin 1	−6·0 (6·5–5·5)	1·9 (1·0–∝)‡	−2·5 (1·5–3·0)
Mahoney	−0·5 (1·0–+0·5)	0·13 (0–0·7)	−0·3 (+0·5–0·5)
Sabin 2	−6·5 (6·5–6·5)	1·6 (1·31–1·8)	—
YSK	−1·0 (1·5–0)	0·20 (0·12–0·5)	—
Sabin 3	−5·5 (6·0–5·0)	75 (100–65)	—
Saukett	−0·5 (1·5–0)	20 (11–25)	—

* $\log_{10}$ difference at 39·8° C. (types 1 and 2) or 40·3° C. (type 3).
† See method for derivation of the values.
‡ ∝ omitted from calculation of average.

The dextran sulphate inhibition test

This was performed by parallel tube titration of each strain in synthetic medium 199 and in this medium incorporating 0·05 % dextran sulphate 2000 (M.W. 2×10^6, Pharmacia). The type 1 virus suspensions were titrated at $\log_{10}$ intervals and two tubes maintained on Medium 199 and two tubes maintained on the dextran medium were inoculated with 0·1 ml. of each dilution. The tubes were incubated at 36° C. and cytopathic effects observed on the 3rd and 6th days. The reduction in titre was calculated from the 6-day end-points. Control titrations of Mahoney and Sabin type 1 virus strains were performed with each batch; less than $10^{0·5}$ reduction in titre of Mahoney virus and more than $10^{5·5}$ reduction in titre of Sabin type 1 virus being accepted.

The range of values obtained with the prototype strains is shown in Table 1.

RESULTS

Considerable variation has been obtained with naturally occurring strains (Tables 2–5). While the values are mainly comparable with those obtained for the prototype wild strains of the same serotype, the results for some strains in each serotype by each test approach those of the vaccine strains.

Table 2. *Type* 1 *strains isolated during* 1953 *from cases of paralytic poliomyelitis*

Passage no.	Laboratory no.	Titre at 37° C. ($\log_{10}$ TCD50/0·1 ml.)	$\log_{10}$ difference at 39·8° C.	Serological index	$\log_{10}$ difference in 0·05% dextran
MK2	4659	6·5	−3·0	0·8	+0·5
	4862	6·0	−4·5	0·5	+0·5
	4863	5·5	−4·0	0·7	+0·5
	5037	6·0	−2·5	0·6	0
	5038	6·0	−2·5	0·1	+1·0
	5045	5·5	−2·0	0·3	0
	5076	6·0	−4·5	0·6	−0·5
	5117	5·5	0	0	+0·5
	5191	6·5	−1·0	0·6	+0·5
	5314	5·5	−4·0	0·6	+1·0
	5382	5·5	−5·5	0	−0·5
	5527	6·5	−5·0	0·6	+1·0
	5747	5·5	−3·0	0·5	0
	6186	6·0	−3·5	0·5	0
	4656	6·5	−2·5	0·5	+0·5
MK3	5343	5·0	−3·5	—	−1·0
MK2	6183	6·5	−1·0	1·0	0
	6808	6·0	−2·5	0·6	0
	166	6·0	−0·5	0·5	+0·5
	182	6·0	−1·0	0·3	0
	274	6·0	−2·0	0·2	+1·5

No single strain, however, shows values for all the marker tests of the vaccine type. Indeed a striking feature is the lack of correlation between the tests. The results of the R.C.T.$_{40}$ test are plotted against the serological indices of the type 1 strains (Fig. 1). The random pattern of the points is striking as is their fairly even distribution over the entire field.

Most of the 1957 type 1 strains were isolated from patients with paralytic poliomyelitis, but they also included a number from cases of aseptic meningitis and non-paralytic poliomyelitis as well as from healthy contacts of cases (Table 3). No characteristic pattern of results was shown by any one of these clinical groups.

The R.C.T. test on type 1 strains shows the most striking degree of variation. If the tests are performed at 39·3° C., however, only a few strains continue to show significant reduction (Table 6).

DISCUSSION

This study was undertaken to obtain a base-line for interpreting the results of genetic marker tests on newly isolated strains of poliovirus and especially for differentiating wild strains from vaccine strains and their progeny. The substantial

degree of variation which has been found with strains from pre-vaccine years suggests that caution is needed in drawing conclusions from the results of these tests. Any one test is insufficient to classify a strain, but with only one or two additional tests a reasonably reliable estimate can be made.

Table 3. *Type* 1 *strains isolated during* 1957

(First passage in monkey kidney tissue culture in all cases.)

Laboratory no.	Clinical illness	Titre at 37° C. ($\log_{10}$ TCD 50/0·1 ml.)	$\log_{10}$ difference at 39·5° C.	Serological index	$\log_{10}$ difference in 0·05 % dextran sulphate
15	P.P.	7·0	−7·0	0·1	−1·0
17	P.P.	7·0	−5·5	0·1	0
20	P.P.	6·0	−0·5	0·2	+0·5
55	P.P.	7·0	−5·0	0·4	+1·0
73	Enc.	6·5	−0·5	0·1	+1·0
77	P.P.	7·5	−4·5	0	0
80	P.P.	6·0	−6·0	0·3	0
88	P.P.	6·5	−4·5	0·2	0
91	P.P.	6·5	−4·0	0	0
123	P.P.	7·5	−4·0	0·4	0
124	A.M.	7·5	−2·0	0·9	+0·5
126	P.P.	7·0	−1·5	0	+0·5
137	P.P.	7·5	−3·0	0·1	+0·5
140	A.M.	6·5	−5·5	0·1	0
142	P.P.	6·0	−4·0	0·2	−1·0
145	P.P.	6·5	−3·0	0·4	0
166	Contact	6·5	−1·0	0·3	0
169	P.P.	6·5	−4·0	0·8	0
170	P.P.	6·5	−4·0	0·3	+0·5
174	P.P.	7·0	−2·0	0·3	0
175	P.P.	6·0	−4·5	0·5	+0·5
198	P.P.	7·0	−5·5	0·6	+0·5
206	A.M. Contact	6·5	−2·5	0	0
209	P.P.	7·0	−5·5	0·5	+1·0
210	P.P.	7·5	−2·5	0·1	−1·0
211	Enc.	7·5	−2·5	0·5	+0·5
212	P.P.	6·5	−1·5	0·2	0
220	P.P.	6·5	−1·0	0·2	−0·5
222	P.P.	6·0	−3·5	0·5	−0·5
245	P.P.	6·5	−1·0	0·6	−0·5
369	N.P.P.	7·5	−2·0	0·3	+0·5
403	P.P.	7·5	−2·0	0·4	0
422	P.P.	7·0	−1·5	0·3	0
425	P.P.	7·5	−2·0	0·4	+0·5

Enc. = encephalitis, N.P.P. = non-paralytic poliomyelitis, P.P. = paralytic poliomyelitis A.M. = aseptic meningitis.

The striking alteration in the behaviour of type 1 strains when the upper temperature in the reproductive capacity temperature test is reduced by only 0·5° C. emphasizes the arbitrary nature of these tests. The original derivation of Sabin's vaccine strain LSc 2ab from Mahoney by successive selection of clones able to grow at suboptimal temperatures (Sabin *et al.* 1954) probably accounts

Table 4. *Type 2 strains isolated in 1953 from cases of paralytic poliomyelitis*

(Second passage in monkey kidney tissue culture in all cases.)

Laboratory no.	Titre at 37° C. ($\log_{10}$ TCD 50/0·1 ml.)	$\log_{10}$ difference at 39·8° C.	Serological index
4659	6·0	−3·0	0
4702	6·5	−6·5	1·6
4895	5·5	−1·0	2·0
4956	5·5	−1·5	0
4957	6·0	−5·0	2·1
5003	6·0	−4·0	5·4
5148	5·5	0	7·0
5196	6·5	−1·0	0·8
5346	5·5	−5·5	0·5
5526	6·0	−6·0	2·5
5599	6·0	−2·0	0·7
5616	6·5	−3·0	0·7
5796	6·5	−2·5	1·5
5993	5·5	0	1·8
4669	6·5	−1·5	2·8
5883	6·0	−1·0	0
6224	6·0	−0·5	0·6
6958	7·0	−2·0	1·2

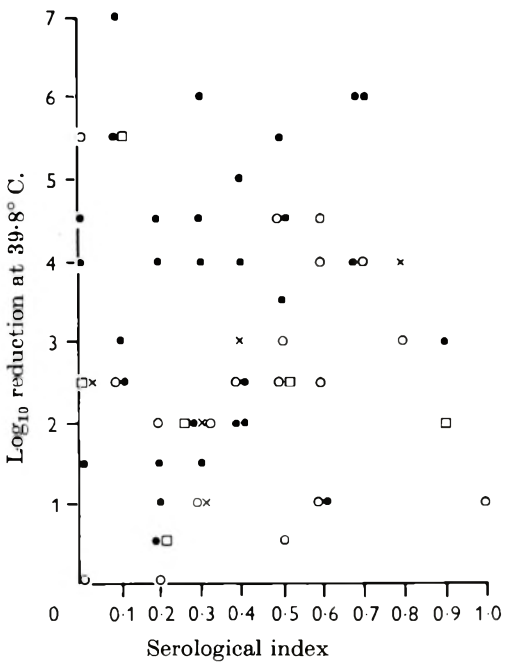

Fig. 1. Correlation of marker tests, type 1. □, 1957 aseptic meningitis; ●, 1957 paralytic polio; ×, 1957 contacts; ○, 1953 paralytic polio.

for the characteristic behaviour of the vaccine strain. There is no evidence in this series that there is any correlation between clinical virulence and the R.C.T.$_{40}$ test for naturally occurring strains.

Table 5. *Type* 3 *strains isolated from cases of paralytic poliomyelitis in* 1953–55

(Second passage in monkey kidney tissue culture in all cases.)

Laboratory no.	Titre at 37° C. ($\log_{10}$ TCD50/0·1 ml.)	$\log_{10}$ difference at 40·3° C.	Serological marker (% difference)
4748	5·5	−5·5	0
4814	7·0	−3·5	−17
5192	5·0	−2·5	+10
43	7·0	−5·5	−33
666	5·5	−2·5	−25

Table 6. *Comparison of the reproductive capacity of type* 1 *strains at* 39·8° *and* 39·3° *C.*

Strain no.	Titre at 36° ($\log_{10}$ TCD50/0·1 ml.)	$\log_{10}$ reduction at 39·8°	$\log_{10}$ reduction at 39·3°
4659	6·0	3·0	0
4863	5·5	4·0	2·0
5076	6·0	4·5	2·5
5314	5·5	4·0	1·0
5382	5·5	5·5	3·0
5527	6·5	5·0	2·0
5745	5·5	3·0	0
6186	6·0	3·5	1·5
5343	5·0	3·5	0·5
15	7·0	7·0	3·0
17	7·0	5·5	1·0
55	7·0	5·0	0
77	7·5	4·5	0·5
80	6·0	6·0	5·5
88	6·5	4·5	4·5
91	6·5	4·0	1·0
123	7·5	4·0	1·0
137	7·5	3·0	4·0
140	6·5	5·5	3·0
142	6·0	4·0	3·0
145	6·5	3·0	2·0
168	6·5	4·0	0
170	6·5	4·0	1·5
174	7·0	7·0	1·0
175	6·0	4·5	4·0
209	7·0	5·5	6·0
222	6·0	3·5	3·5
Mahoney	6·5	0·5	0
Sabin 1	6·0	6·0	4·5

Two points of practical importance emerge. First, the R.C.T.$_{40}$ test may be useful as a marker test in epidemiological studies, when subgrouping of naturally occurring strains may be attempted by varying the temperature over the range 39–40° C. Secondly, very strict temperature control is necessary to obtain reproducible results which can be compared with those in other laboratories.

The variation found with the serological and dextran markers is similar to that described (Karzon, Pollock & Barron, 1959) for other enteroviruses.

SUMMARY

The properties of naturally occurring poliovirus strains of each serotype have been studied using three marker tests: the R.C.T.$_{40}$ test, intratypic serodifferentiation and, for type 1 strains only, inhibition by dextran sulphate.

The results show that while most strains resemble the prototype strain of the same serotype, considerable natural variation exists, especially in the ability to grow at temperatures between 39° and 40° C.

No correlation has been found between the results of the tests on individual strains and it is concluded that no single test is sufficient to characterize a strain.

I wish to acknowledge the helpful advice of Dr A. D. Macrae, Virus Reference Laboratory, and also to thank Miss Marjorie Bennett for her technical assistance.

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Genetic marker studies of poliovirus

II. Strains isolated from cases of poliomyelitis associated with the administration of live attenuated vaccine

By YVONNE E. COSSART

Virus Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

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The experience of a decade of vaccination against poliomyelitis demonstrates that the disease can be prevented by either formalin-inactivated Salk-type vaccine or live attenuated Sabin vaccine. The practical advantages of live vaccine have led to its use for mass vaccination in many countries, and epidemiological studies have shown that its large-scale use is very safe (Galbraith, 1963; Gelfand, 1963).

However, studies of the viruses excreted by recipients of live vaccine have revealed that the laboratory characters of the Sabin strains, especially type 3, revert towards those of the naturally occurring strains after quite short periods of intestinal passage (Magrath, Boulger & Hartley, 1964).

The theoretical risk to those vaccinated and to their contacts is thus not entirely negligible. For this reason the Public Health Laboratory Service Poliomyelitis Surveillance Committee has sought all cases of poliomyelitis associated with the administration of vaccine (Miller & Galbraith, 1965). The criteria adopted for vaccine association are administration of oral vaccine to the patient himself in the 28 days preceding the onset of symptoms, or to a household contact within 60 days of onset.

During 1962, the first year of live virus vaccination in England and Wales, 18 paralytic cases were notified and faecal specimens were examined from 17 of these. Strains of poliovirus were obtained from 15 and of these 13 were available for the present study. The remaining 2 faecal extracts failed to yield virus when re-isolation was attempted after storage. In addition a number of other strains were tested: from household contacts of the above cases, from non-paralytic reactions attributed to vaccination, and sporadic strains apparently unrelated to vaccination. Tables 1–3 list the strains and the clinical details.

An attempt has been made to group these strains into wild and vaccine categories on the results of genetic marker tests. No marker test so far proposed corresponds exactly to the monkey neurovirulence test while the exact relation of even the latter to human virulence is not finally decided (Pavilanis *et al.* 1964). It seemed desirable then to choose tests to cover as wide a range of virus properties as possible. On the other hand it was necessary to limit the tissue culture and labour used for the tests.

The reproductive capacity temperature-marker test (R.C.T.$_{40}$) (Lwoff, 1962) and

the intratypic serodifferentiation test (Wecker, 1960) have been applied to strains of all three serotypes; and the dextran sulphate inhibition test (Cossart, 1966) has in addition been used for type 1 strains. The R.C.T.$_{40}$ test measures a feature of the growth cycle of the virus strains while the serological and dextran tests depend on the properties of the protein coat of the particles themselves.

Table 1. *Poliovirus type* 1 *strains*

Origin	Laboratory no.	Vaccine history	Clinical details
Colindale	284	Nil	Paralytic poliomyelitis
	285	Nil	Aseptic meningitis
	286	Nil	Aseptic meningitis
Bristol	308	Self: 14th day before onset	
Leicester	312	Nil	
Cardiff	315	Self: day of onset	
	316	Nil	
	318	Nil	
	319	Self: 4th day before onset	Paralytic poliomyelitis
	427A		
	427B		
	427C		
	320		
	321		
	323	Self: 14th day before onset	
	430	Self: 1st day before onset	
	431A	Self: 5th day before onset	
	431C	Self: 5th day before onset	
	431D	Self: 5th day before onset	
Guildford	568	Sibling: 22nd day before onset	Fatal encephalitis
	569	Self: 22nd day before onset	Healthy
	570	Sibling: 22nd day before onset	Healthy

Table 2. *Poliovirus type* 2 *strains*

Origin	Laboratory no.	Vaccine history	Clinical details
Leicester	399	Sibling: 40 days before onset	Weakness in arm following smallpox vaccination to self
Cardiff	426A	Self: 3rd day before onset	Paralytic poliomyelitis
	B		
	C		
	D		
	E		
Colindale	287	Self: 1st day before onset	Diarrhoea

MATERIALS AND METHODS

The R.C.T.$_{40}$ test, intratypic serodifferentiation and the dextran sulphate test were performed by the techniques which have been described fully in the previous paper (Cossart, 1967).

The range of values obtained with the prototype strains are given in Table 4.

Table 3. *Poliovirus type 3 strains*

Origin	Laboratory no.	Vaccine history	Clinical details
Southend	263	Child: 13th day before onset	Paralytic poliomyelitis (mother of 265, 6, 8 and 9)
	264	Child: 13th day before onset	
	265	Self: 13 days before onset	Children of 263
	266	Sibling: 13 days before onset	
	268	Sibling: 13 days before onset	
	269	Sibling: 13 days before onset	
Guildford	357	Self: 6 weeks before onset	Bulbar poliomyelitis
Manchester	382	Self: 14 days before onset	Paralytic poliomyelitis
Bradford	386	Self: 17 days before onset	Paralytic poliomyelitis
	388	Self: 24 days before onset	Child of paralytic poliomyelitis
Colindale	395	? H.C.* ? interval	Fatal encephalitis
	414	Sibling: 21st day before onset	Paralytic poliomyelitis

* H.C. = household contact.

Table 4. *Results of marker tests on prototype strains given as the average of five determinations with the range of values shown in brackets*

Strain	R.C.T.$_{40}$ ($\log_{10}$ reduction*)	Serological index†	Dextran marker ($\log_{10}$ reduction)
Sabin 1	6·0 (6·5–5·5)	1·9 (1·0–∞)‡	−2·5 (1·5–3·0)
Mahoney	0·5 (1·0–+0·5)	0·13 (0–0·7)	0·3 (+0·5–0·5)
Sabin 2	6·5 (6·5–6·5)	1·6 (1·31–1·8)	—
YSK	1·0 (1·5–0)	0·20 (0·12–0·5)	—
Sabin 3	5·5 (6·0–5·0)	75 (100–65)	—
Saukett	0·5 (1·5–0)	20 (11–25)	—

* $\log_{10}$ reduction at 39·8° (types 1 and 2) or 40·3° C. (type 3).
† See method for derivation of the values.
‡ ∞ omitted from calculation of average.

RESULTS

Tables 5–7 list the results of the three marker tests. The number of each serotype isolated from the paralytic vaccine-associated cases is: Type 1, 8; Type 2, 1; Type 3, 4. In the United States in a similar series Gelfand (1963) reported the isolation of 8 type 1 strains, no type 2 strains and 13 type 3 strains while Furez, Armstrong, Moreau & Nagler, (1964) found that 12 of 17 vaccine-associated cases in Canada in 1962 excreted type 3 viruses only. The predominance of type 1 in England and Wales may be due to the use of oral vaccine for controlling an outbreak of type 1 poliomyelitis in South Wales. Seven of the eight type 1 strains were from this area.

These seven strains closely resembled each other and the two strains (316 and 318) from unvaccinated cases of paralytic poliomyelitis in the same area. The four other type 1 strains (284, 285, 286 and 312) with no association with vaccine are all similar to the South Wales strains. The most distinctive feature of their behaviour is the depression of growth at 39·8° C. in many instances to values in the vaccine range.

As both the serological and dextran markers gave results distinctly of the wild type it was decided to repeat the reproductive capacity temperature test using 39·3° C. as the upper temperature. The results are shown in Table 8.

Table 5. *Results of marker tests: type* 1

Laboratory no.	Titre at 36° C.	R.C.T.$_{40}$ $\log_{10}$ difference at 39·8° C.	Serological index	Dextran marker $\log_{10}$ difference
284	7·0	−5·5	0·63	+1·0
285	7·0	−6·0	0·61	+0·5
286	6·0	−4·5	0·64	0
*308	5·5	−5·5	1·05	0
312	6·5	−4·5	0·35	0
*315	6·0	−3·0	0·66	0
316	6·0	−3·5	0·35	0
318	6·0	−3·5	0·72	−0·5
*319	6·0	−2·0	1·0	−1·0
*427A	7·0	−5·5	0·66	0
*427B	7·0	−5·5	0·28	0
*427C	6·5	−1·5	0·50	0
*320	6·5	−5·0	0·80	+0·5
*321	6·5	−5·0	0·60	+0·5
*323	6·0	−3·0	0·78	0
*430	6·0	−6·0	0·72	−0·5
*431A	6·0	−6·0	0·93	−0·5
*431C	5·5	−5·5	1·21	0
*431D	7·0	−6·5	0·66	−0·5
568	7·5	−7·5	∝	−2·0
569	6·5	−6·5	19·0	−2·0
570	6·0	−6·0	∝	−1·5

* Vaccine associated cases of paralytic poliomyelitis.
Strains isolated from a single patient are grouped together by a brace.

Table 6. *Results of marker tests: type* 2

Laboratory no.	Titre at 36° C.	R.C.T.$_{40}$ ($\log_{10}$ difference at 39·8° C.	Serological index
399	6·5	−6·5	2·3
*426A	5·5	−6·0	1·2
*426B	6·0	−5·5	∝
*426C	6·5	−5·0	1·6
*426D	6·0	−6·0	2·2
*426E	6·0	−5·5	3·4
287	6·0	−4·5	1·5

* Vaccine-associated cases of paralytic poliomyelitis.
Strains isolated from a single patient are grouped together by a brace.

The reduction of only 0·5° C. restored the growth of most of the strains almost to the titre at 36° C. Similar behaviour has also been found by Magrath (1966) with some wild type 1 strains isolated from cases of paralytic poliomyelitis in 1953 and 1957. In contrast, however, strains 568, 569 and 570 remain almost

completely inhibited as does Sabin type 1 virus. These three strains were thought to be of vaccine origin on clinical grounds, and both serological and dextran markers also suggest this. Strains 430 and 431, however, fail to give clear-cut results and have been classed as intermediate on the basis of the three tests used.

All the type 2 strains are of vaccine type in both marker tests. This was expected

Table 7. *Results of marker tests: type* 3

Laboratory no.	Titre at 36° C.	R.C.T.$_{40}$ ($\log_{10}$ difference) at 40·3° C.	Serological index
*263	6·0	−1·5	40
*264	6·5	−3·5	28
265	6·0	−1·0	50
266	6·5	−4·5	92
268	5·5	0	54
269	7·5	−2·0	33
357	5·5	−4·0	44
*382	5·5	−1·5	21
*386	6·0	−2·0	25
388	5·0	−2·5	66
395	5·5	−2·5	8
*414	6·0	−2·0	11

* Vaccine associated cases of paralytic poliomyelitis.
Strains isolated from a single patient are grouped together by a brace.

Table 8. *Comparison of two different upper temperatures in the* R.C.T.$_{40}$ *test for type* 1

Laboratory no.	Titre ($\log_{10}$) at 36° C.	Log_{10} reduction at 39·8° C.	Log_{10} reduction at 39·3° C.
284	7·0	5·5	0
285	7·0	6·0	0·5
286	6·0	4·5	0
308	5·5	5·5	3·0
312	6·5	4·5	0·5
315	6·0	3·0	2·0
316	6·0	3·5	1·5
318	6·0	3·5	2·0
319	6·0	2·0	0
427A	7·0	5·5	0
427B	7·0	5·5	2·0
427C	6·5	1·5	1·5
320	6·5	5·0	0·5
321	6·5	5·0	2·5
323	6·0	3·0	2·0
430	6·0	6·0	4·0
431A	6·0	6·0	3·0
431C	5·5	5·5	2·5
431D	7·0	6·5	4·0
568	7·5	7·5	4·5
569	6·5	6·5	4·0
570	6·0	6·0	4·0
Mahoney	6·5	0·5	0
Sabin 1	6·5	6·0	4·0

from the clinical diagnosis for 399 and 287, but 426 is a case of paralytic poliomyelitis in the same South Wales outbreak from which most of the type 1 strains were derived. The persistent excretion of type 2 virus is unexpected. If the illness was really due to a wild type 1 strain it does not seem likely that it would be displaced so thoroughly by a single Sabin strain given 3 days before the onset of symptoms (Feldman, Halquin & Gelfand, 1964). No serological evidence is available to clarify the position.

The type 3 strains show a graduation of results throughout the range. Numbers 263 and 264 are from an adult case of severe paralytic poliomyelitis from whom, however, no satisfactory serological data are available. One of her children (265) was given oral vaccine 13 days before the onset of her own illness. Strains 266, 268 and 269 were isolated from the stools of the three other children who were not vaccinated. One child was excreting a typical vaccine strain (266) but the strains from the others, including the vaccinated child and the patient herself, show varying degrees of reversion to values well within the wild range. This provides a fairly clear example of a vaccine strain reverting to virulence. The three other vaccine-associated cases of paralysis (382, 386 and 414) have values in the wild range for both markers.

Number 388, the strain from a vaccinated child of an adult case of paralysis from whom no virus was isolated, has intermediate values, as has 357, a clinically doubtful case of bulbar poliomyelitis occurring 6 weeks after vaccination. A case of encephalitis (395) with only a possibility of contact with vaccine is of wild type in both tests.

DISCUSSION

It is seen that only one case of vaccine-associated paralytic poliomyelitis (no. 426) yielded a strain with *in vitro* vaccine characters, and there is a suspicion that this type 2 strain may not have been responsible for the illness.

The type 1 strains can be grouped with some confidence into wild and vaccine groups, only 2 of the 18 cases yielding intermediate strains. The South Wales strains form a homogeneous group, whether from vaccinated cases or not, and their distinctive behaviour in the R.C.T.$_{40}$ test suggests they should all be regarded as of wild origin.

It would also seem that the upper temperature used in the R.C.T.$_{40}$ test should be reduced to 39·3° C. when testing type 1, since above this temperature some wild strains may fail to grow. Variation in this temperature over the range 39–40° C. may reveal differences between wild strains from different sources and could be a useful epidemiological tool.

The type 3 strains studied include some from a single family in which reversion of the vaccine strain to clinical and laboratory virulence probably occurred. The possibility that the unvaccinated siblings and parent were harbouring wild type 3 strains with such a range of properties seems remote; no other type 3 strains were isolated in the area over the period studied. With the tests used the probable origin of the three type 3 strains from vaccine-associated cases which behave like wild strains in the marker test remains in doubt.

Of the strains from 13 patients obtained through epidemiological surveillance, *in vitro* marker tests were able to class 6 as being unrelated to vaccine and 3 others as also wild but of uncertain origin. One strain was of vaccine type, two had intermediate characters and reversion to virulence seemed probable in the strains from the remaining case and her family.

SUMMARY

Strains of poliovirus were obtained from 13 of the 18 persons in England and Wales with paralytic episodes after administration of oral vaccine in 1962. They have been studied using three marker tests: the R.C.T.$_{40}$ test, intratypic serodifferentiation and inhibition by dextran sulphate. For comparison a number of strains from subjects with non-paralytic vaccine-associated reactions and from patients with paralytic poliomyelitis not related to vaccine were also tested.

Of the eight patients excreting type 1 strains seven came from South Wales where an outbreak was in progress. They all resemble naturally occurring strains from the outbreak in growing at 39·3° but not at 39·8° C.

Only one subject excreted type 2 virus which was of vaccine type.

The type 3 strains included a series from a family group where a range of results from vaccine to the wild range was obtained. Three other patients with vaccine-associated paralysis excreted type 3 strains with the characteristic of naturally occurring strains.

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With 1 *plate*

Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs

By R. F. W. GOODWIN, ANNETTE P. POMEROY and P. WHITTLESTONE
School of Veterinary Medicine, University of Cambridge

(*Received* 3 *November* 1966)

Enzootic pneumonia of pigs is a common disease in Britain; it has also been reported from many other countries and it will probably become even more apparent as the trend to keep pigs in larger numbers in close indoor confinement continues. The essence of the problem in the field derives from the fact that the pneumonic lesions may persist throughout the main growing life of the pig, during which time the affected animal usually remains infective to other pigs. Although rarely fatal, the condition is often associated with a reduction in growth rate and the cumulative economic loss is so great, both nationally and internationally, that enzootic pneumonia has been described as the most important disease of pigs today.

From one strain of enzootic pneumonia (the J strain) studied in this laboratory, Goodwin & Whittlestone (1963) grew an agent in tissue culture which induced the typical disease. Later, this agent was grown in an acellular liquid medium, from which it likewise induced enzootic pneumonia experimentally (Goodwin & Whittlestone, 1964, 1966). By this time it was widely assumed that the causal agent of enzootic pneumonia was a *Mycoplasma*, but the problem of growing the agent on solid medium and reproducing the disease with the resulting colonies remained. Although mycoplasma colonies could occasionally be isolated on solid medium from our liquid-medium cultures at this stage, we could not passage these colonies on solid medium. Eventually, however, by using improved cultural methods, enzootic pneumonia was induced with mycoplasma colonies that, after several passages on solid medium, were at least a 10^{-15} dilution of the first seed inocula (Goodwin, Pomeroy & Whittlestone, 1965), the mycoplasma being named *Mycoplasma suipneumoniae*. In the present paper, the pathogenic role of this mycoplasma forms one part of a fuller description of the organism.

MATERIALS AND METHODS

Strains of mycoplasma

M. suipneumoniae was derived from the J strain of enzootic pneumonia, which originated from a field outbreak of the disease. This strain has been shown to be consistently infective during serial transmissions in pigs over a period of several years and it regularly contained pleomorphic organisms. One of these transmitted pneumonias yielded an organism in tissue cultures (Goodwin & Whittlestone,

1963), from which the disease was induced in pig 2206 (Goodwin & Whittlestone, 1965). Pneumonic material from pig 2206 was inoculated into plasma-clot cultures prepared from the normal lung of a hysterectomy-produced colostrum-deprived pig, and the fluids from these cultures, harvested after 3 days, were used to initiate the passages on solid medium.

Mycoplasma strain 603 was isolated from a field case of enzootic pneumonia but has not itself induced the disease experimentally (Goodwin & Whittlestone, unpublished).

The sources of the various other mycoplasmas used in this work are shown in the appropriate parts of the text.

Cultural media

Our original solid medium, which had been used for producing the pig inocula, contained lung broth. Subsequently, the lung broth was replaced by Hartley's broth (made with ox heart). In limited comparative experiments, the Hartley's-broth medium produced better growth of *M. suipneumoniae*; furthermore, because this gave a clearer medium, it was used for nearly all the growth-inhibition tests. To make about 100 ml. of medium, 1 g. of Oxoid Ionagar no. 2 was added to 40 ml. of balanced salt solution (Hanks's with 0·001 % phenol red) at 56° C. and autoclaved at 1·2 atm. (gauge pressure) for 15 min. The following sterilized constituents were then warmed to 56° C. and mixed together before adding to the Hanks's solution plus agar at 56° C.: either lung broth or Hartley's broth (30 ml.), serum from enzootic-pneumonia-free pigs (20 ml.) and 5 % lactalbumin hydrolysate (10 ml.). The final medium also contained penicillin (200 units/ml.), thallium acetate (1/8000) and yeast extract (0·5 %). The yeast extract was prepared as described by Herderscheê (1963). The lung broth was made by mincing lung from hysterectomy-produced colostrum-deprived pigs and leaving this overnight at 4° C. in twice the weight of water; this suspension was then filtered through double gauze, simmered for 15 min., filtered again through gauze, and centrifuged (900 *g* for 20 min.), the supernatant fluid being autoclaved thereafter (0·5 atm., gauge pressure, for 30 min.) and tested for sterility. With *M. hominis* type I, *M. orale* type II and *M. pulmonis*, which did not grow readily on this medium, the medium used was that of Taylor-Robinson, Somerson, Turner & Chanock (1963).

The main liquid medium used was the same as the solid medium containing Hartley's broth, but without the agar. For the pig infectivity work, however, the liquid medium contained lung broth instead of Hartley's broth. The use of an earlier liquid medium (Goodwin & Whittlestone, 1964, 1966) is referred to in the text.

Samples for routine bacteriology were plated on horse-blood agar, chocolate agar and Edward's medium (with 1/8000 thallium acetate); incubation was aerobic at 37° C.

Routine incubation of the mycoplasma cultures was as follows: the liquid-medium tubes were rolled at 37° C.; the solid-medium plates were incubated in a moist atmosphere containing CO_2 (5–10 % in air).

Antisera

The sera used in the immuno-fluorescent work and for precipitation in agar gel (rabbit sera R 3 and R 4, both against *M. suipneumoniae*, and R 14, against mycoplasma 603; pig sera P 2268 and P 2269, both against *M. suipneumoniae*) were made before *M. suipneumoniae* could be grown on solid medium. The mycoplasmas were therefore grown either in pig-lung-monolayer cultures (Goodwin & Whittlestone, 1963) or cell-free fluids (Goodwin & Whittlestone, 1964). The sera used in the growth-inhibition and metabolic-inhibition tests (rabbit sera R 2 and R 7, both against *M. suipneumoniae*, R 43 against *M. hyopneumoniae* of Maré & Switzer (1965) and R 1 against mycoplasma 603) were made using mycoplasmas grown in the liquid medium containing Hartley's broth: the antigens for rabbits R 7, R 43 and R 1 were grown in liquid medium after at least four serial single-colony passages on solid medium. In all cases the antigen was centrifuged, washed several times in phosphate-buffered saline (PBS) and resuspended in PBS.

The rabbits, after bleeding, received one subcutaneous injection of antigen with Freund's complete adjuvant, followed by intravenous injections, except that the first inoculation of rabbit R 2 was intradermal, and rabbits R 7 and R 43 received an intramuscular injection (with incomplete Freund's adjuvant) at the same time as their final intravenous inoculations. The pigs received live cultures of *M. suipneumoniae* in tissue-culture fluids intranasally, followed by subcutaneous injections of antigen with Freund's complete adjuvant. For precipitation in agar gel, the rabbit sera were absorbed with normal pig serum; for immunofluorescence they were absorbed with pig lung-liver powder (Nairn, 1962). The globulins in the pig sera were precipitated with sodium sulphate (Marrack, Hoch & Johns, 1951) and reconstituted in one fifth of the original volume.

The antiserum to rabbit gamma-globulin was prepared in a goat by injecting alum-precipitated globulin intramuscularly; the globulins were concentrated as above.

The sera supplied by other laboratories are identified in the text. All sera were stored at about $-20°$ C.

Serological techniques

Growth inhibition

The method of Clyde (1964) was used, except that the plates were inoculated either directly with colonies from solid medium or with a suspension of such colonies and, secondly, the disks were soaked in 0·01–0·025 ml. of antiserum, allowed to dry (Stanbridge & Hayflick, in preparation), and stored at $-20°$ C. until required.

Metabolic inhibition

The method of Taylor-Robinson, Purcell, Wong & Chanock (1966) was used.

Precipitation in agar gel

The double-diffusion method of Ouchterlony (1964) was used.

Immuno-fluorescence

Control and infected cover-slips were rinsed in PBS and fixed in dry acetone for 5 min.; they were then stained immediately or stored at −20° C., or lower temperatures, until required. Acetone-fixed smears of concentrated antigen were also used.

Conjugation with fluorescein isothiocyanate (Gurr) was according to Marshall, Eveland & Smith (1958), the conjugate being purified by filtration on a G 25 coarse-grade Sephadex column. The fluorescent filtrate was concentrated by dialysis against Carbowax (Kohn, 1959) and then absorbed with reconstituted pig lung-liver powder by shaking at room temperature for 1 hr. (100 mg./ml. of serum); the powder was then removed by centrifugation (30,000*g*). Normal goat serum was conjugated to lissamine rhodamine B and added to the anti-rabbit-globulin conjugate (ratio 1:5) as a counterstain. Only pig serum P 2268 was conjugated for use in the direct method.

Staining (37° C. for 30 min.) was as described by Nairn (1962); in the indirect method the initial incubation with rabbit serum was at 37° C. for 1 hr. Preparations were examined with Leitz Ortholux equipment; the UV light source was HBO 200 W, the screening filter was UG 1 (1·5 mm.), and a dark-field condenser was used. Assessment of the number of organisms, and their colour and brightness compared with the background, was made without the observer knowing the identity of the specimens.

Pig inoculations

All the pigs were hysterectomy-produced, colostrum-deprived animals. They were reared and infected under conditions of strict isolation in a specially designed building: each cubicle is approached through an ante-room in which protective clothing is put on, and before each experiment all the equipment in the cubicles is first steamed and then fumigated with formalin within the cubicle itself. The pigs that were infected with cultures and the pigs used in the first pig-passages were 3–5 weeks old; the pigs used in the second pig-passage were 24 weeks old; and the pigs used to produce sera P 2268 and P 2269 were 7 weeks old. All the attempts to produce enzootic pneumonia were by intranasal inoculation; the dose of lung tissue was 6–8 ml. of a 10^{-1} suspension in broth.

Enzootic pneumonia was diagnosed by the macroscopic appearance of the lesions, the histological picture, and the presence of pleomorphic organisms in touch preparations (Whittlestone, 1967).

RESULTS

General characteristics of Mycoplasma suipneumoniae

On the solid media incorporating lung-broth or Hartley's broth, minute colonies (*ca.* 20–100 μ diam.) were first seen at 3–5 days; these enlarged to a maximum diameter of 400 μ at 7–10 days, when they appeared as convex mycoplasma-type colonies, devoid of a central nipple (Plate 1, fig. 1). There was considerable variability in colony size. No growth into the medium was observed. Colonies

could not be passaged on Edward's medium; nor could they be grown on our standard medium anaerobically. In liquid medium, growth was associated with the production of acid and a faint opalescence was occasionally seen in the best cultures. In such cultures the mycoplasma appeared to be non-motile. On some occasions, and particularly when liquid medium was seeded with colonies from solid medium, the organisms did not adhere to the cover-slips, so that the growth assessments were made initially from the pH change, coupled with the examination of stained, dried drops of the liquid cultures. The mycoplasma appeared to grow as well when the Hanks's solution contained no glucose.

Whatever the medium (solid media, liquid media, tissue cultures, or the living pig), the individual elements of the mycoplasma were very pleomorphic, but in each of these culture systems the organism tended to grow in a manner characteristic for the system concerned. Thus, in touch preparations from lung, ring and bipolar forms (0·5–0·8 μ diam.) predominated and the organism occurred singly or in groups. In tissue cultures (Goodwin & Whittlestone, 1963) the organism was usually in diffuse groups, mainly as cocci (*ca.* 0·5 μ diam. and commonly in short chains) or rings (up to 3 μ diam.) containing a single coccus-like structure. In liquid media (Goodwin & Whittlestone, 1966), the main forms were cocci strung on fine branching filaments (0·1 μ diam.), or globular structures, usually in colonies; under the best conditions, as in the medium containing Hartley's broth, growth was more confluent and the globular structures were both larger (up to 16 μ) and more plentiful (Plate 1, fig. 2).

The mycoplasma grew well in liquid media and on solid media in the presence of thallium acetate (1/8000) and penicillin (200 units/ml.), whereas it was inhibited on solid medium by tetracycline (10 μg. disk), in a zone just over 3 cm. wide.

Serological tests

Growth inhibition

Rabbit sera R 2 and R 7, prepared against *M. suipneumoniae*, both gave good results in this test against the homologous culture, as also did the serum prepared by us against *M. hyopneumoniae* (Maré & Switzer); the zones of inhibition varied from 5 to 11 mm., measured from the edge of the disk to the beginning of colony growth. The sera prepared against *M. suipneumoniae* inhibited *M. hyopneumoniae*, and the serum prepared against the latter organism inhibited *M. suipneumoniae* to about the same extent.

The mycoplasmas listed in Table 1 were all inhibited by their respective homologous antisera but not by antiserum R 2 or R 7 prepared against *M. suipneumoniae*; also, in each case, the respective homologous antiserum to the mycoplasma named failed to inhibit a cloned culture of *M. suipneumoniae*. The sources of the cultures and antisera are shown.

The mycoplasmas listed in Table 2 were not inhibited by antiserum prepared against *M. suipneumoniae*. Although cultures of *M. suipneumoniae* were not inhibited by antisera prepared against these other mycoplasmas, no inhibition was obtained when these same antisera were used against their homologous cultures. The sources of the cultures are shown.

The mycoplasmas listed in Table 3 were not inhibited by antiserum prepared against *M. suipneumoniae*. No homologous antisera were available. The sources of the cultures are shown.

Table 1. *Mycoplasmas differing from* Mycoplasma suipneumoniae: *homologous system working*

Name of organism	Source and identity of culture	Source and identity of antiserum
M. hyorhinis F*	Dinter, Danielsson & Bakos (1965)	Dinter, Danielsson & Bakos (1965)
M. granularum B 2		
B 3		
B 4		
B 6		
M. pneumoniae	National Institutes of Health (Eaton FH)	Hayflick (Eaton FH)
M. salivarium	Taylor-Robinson (PG 20)	Hayflick (H 145)
M. orale I	Taylor-Robinson (CH 19299)	National Institutes of Health (CH 19299)
M. orale II*	Taylor-Robinson (CH 20247)	Taylor-Robinson (CH 20247)
M. fermentans	Taylor-Robinson (PG 18)	Edward & Leach (PG 18)
M. hominis I*	Taylor-Robinson (PG 21)	National Institutes of Health (PG 21)
M. pulmonis	Edward & Leach (PG 34)	Hayflick (Negroni)
*M. arthritidis**	Edward & Leach (Campo, PG 27)	Edward & Leach (PG 6)
M. mycoides var. *mycoides*	Edward & Leach (PG 1)	Edward & Leach (PG 1)
M. laidlawii A	Edward & Leach (PG 8)	Hayflick (41)
M. laidlawii B	Edward & Leach (PG 9)	Hayflick (41)
*M. agalactiae**	Edward & Leach (PG 2)	Edward & Leach (PG 2)
M. bovirhinis	Edward & Leach (Bovine group 4, PG 43)	Edward & Leach (PG 43)
Bovine group 7	Edward & Leach (PG 50)	Edward & Leach (PG 50)
Bovine group 8	Edward & Leach (PG 51)	Edward & Leach (PG 51)
M. canis	Edward & Leach (PG 14)	Edward & Leach (PG 14)
M. gallisepticum	Edward & Leach (X 95, PG 31)	Chu (A 5969)
M. gallinarum	Edward & Leach (PG 16)	Chu
Iowa 695	Roberts (2)	Chu (Iowa 695)

* Weak result; that is, not a complete absence of colonies in the zone of inhibition.

Table 2. *Mycoplasmas differing from* Mycoplasma suipneumoniae: *homologous system not working*

Name of organism	Source and identity of culture
M. hyorhinis S 7	Dinter, Danielsson & Bakos (1965)
GDL	Edward & Leach (PG 44)
M. bovigenitalium	Edward & Leach (B 2, PG 11)
M. neurolyticum	Edward & Leach (Sabin A, PG 39)
M. spumans	Edward & Leach (PG 13)
M. 'agalactiae var. *bovis'*	Edward & Leach (Bovine group 5, PG 45)
Bovine group 6	Edward & Leach (PG 49)
M. mycoides var. *capri*	Edward & Leach (PG 3)

Table 3. *Mycoplasmas differing from* Mycoplasma suipneumoniae: *no homologous serum available*

Name of organism	Source and identity of culture
M. iners	Edward & Leach (PG 30)
M. maculosum	Edward & Leach (PG 15)
M. meleagridis	Roberts
M. anatis	Roberts (4)
WR 1	Roberts (3)
186	Roberts (6)
658	Roberts (7)
Avian 8	Roberts

Table 4. *Summary of reactions in agar gel and with immuno-fluorescence*

	Reaction in agar gel					Titre with immuno-fluorescence		
	Pig antisera		Rabbit antisera			Pig antiserum	Rabbit antisera	
Antigen	P 2268	P 2269	R 3	R 4	R 14	P 2268	R 3	R 14
M. suipneumoniae	+	+	+	+	−	1/160	1/320	−
Mycoplasma 603	−	−	−	−	+	NT	−	1/640

NT = not tested.

NOTE. Serum R 14 was prepared against mycoplasma 603; all the other sera were prepared against *M. suipneumoniae*.

Relationship of mycoplasma 603 *with other porcine mycoplasmas.* Mycoplasma 603 and *M. hyorhinis* S 7 were not inhibited by their respective homologous sera in the growth-inhibition test; mycoplasma 603 was also not inhibited by sera prepared against the following porcine strains: *M. suipneumoniae, M. granularum, M. hyorhinis* S 7, *M. hyorhinis* F, B 2, B 3, B 4 and B 6. The last seven strains were from Dinter, Danielsson & Bakos (1965). Nor did rabbit serum R 1 prepared against mycoplasma 603 inhibit any of these eight mycoplasmas, except for *M. hyorhinis* F. Thus, by this test, there seems to be some relationship between mycoplasma 603 and *M. hyorhinis* F.

Metabolic inhibition

M. suipneumoniae and *M. hyopneumoniae* were indistinguishable in the metabolic-inhibition test: both organisms were inhibited by rabbit serum R 2 (against *M. suipneumoniae*) and rabbit serum R 43 (against *M. hyopneumoniae*) to titres of 1/640–1/1280. Both the pre-inoculation rabbit sera did not inhibit the metabolism of either mycoplasma at the lowest dilution (1/40).

Mycoplasma 603 and *M. hyorhinis* (Edward & Leach) were indistinguishable by the same test, in that both organisms were inhibited by rabbit serum R 1 against mycoplasma 603 to a titre of 1/2560. No inhibition was obtained with the pre-inoculation serum at a dilution greater than 1/40.

Precipitation in agar gel

The main findings in the double-diffusion tests are summarized in Table 4.

Antisera R 3 and R 4 against *M. suipneumoniae* gave at least three precipitation lines against the homologous mycoplasma grown either in tissue cultures or in cell-free fluids. No lines were obtained with cells from control cultures centrifuged at 30,000 *g* for 60 min., or with mycoplasma 603.

Concentrated globulin fractions of the pig sera gave rise to two precipitation lines on reaction with *M. suipneumoniae* and these seemed to be continuous with one of the lines arising from the reactions between *M. suipneumoniae* and rabbit antiserum R 3. These concentrated fractions gave no lines with either centrifuged cells from control cultures or with mycoplasma 603.

Serum R 14, prepared against mycoplasma 603, formed a single line against mycoplasma 603; this line crossed those formed between *M. suipneumoniae* and its homologous antisera, showing that the two antigens were unrelated. No lines were obtained with centrifuged cells from control cultures.

Immuno-fluorescence

In general, it was easier to see the mycoplasmas in the fluorescent preparations than in Giesma-stained cover-slips. In the direct method, *M. suipneumoniae* grown in both living tissue cultures and cell-free fluids could be stained to a pig-serum titre of 1/160; no such staining was seen with control coverslips. In the indirect method, *M. suipneumoniae* grown in the same two systems could be stained to a rabbit-serum titre of 1/320. Negative results were obtained in parallel tests with pre-inoculation rabbit sera, with control cover-slips, with the conjugate alone, and with mycoplasma 603.

Mycoplasma 603 was stained to a rabbit-serum titre of 1/640. In parallel tests with pre-inoculation serum, conjugate alone, and *M. suipneumoniae*, no such staining occurred.

All these results are summarized in Table 4.

Production of enzootic pneumonia in pigs

Two main attempts were made to induce enzootic pneumonia with colonies of *M. suipneumoniae*. The general plan was to passage the agent on solid medium and inoculate pigs with several doses of the final mycoplasma colonies washed from this medium. In case such colonies were less effective at inducing pneumonia than the same agent grown in liquid medium, the inoculum prepared from the solid medium was also inoculated into liquid medium and, after passage in this medium, further pig inocula were prepared which were given to other pigs in each of the two experiments. The details concerning the preparation of all these inocula have been given elsewhere (Goodwin *et al.* 1965).

In the first experiment, the mycoplasma was maintained on solid medium for 42 days and the dilution of the inoculum for the first solid-medium passage in the final material given to the pigs was at least 10^{-15}. None of the three pigs that received the mycoplasma directly from solid medium developed pneumonia. Two

of the three pigs that received the mycoplasma after subsequent passage in liquid medium, however, developed typical enzootic pneumonia. Two control pigs, which had been inoculated with the same batch of liquid medium as that which had been used to prepare the infected inocula, were killed at about the same time: they showed no sign of pneumonia. No significant bacteria were isolated from the lungs of any of the pigs in this experiment. Thus, the disease was produced only after passaging the mycoplasma from solid medium further in liquid medium, despite the fact that it had thereby been passaged outside the pig for a longer period.

In the second experiment, fewer passages were made on solid medium before gaining the required dilution of the primary solid-medium inoculum (at least 10^{-15}); because of this, the mycoplasma was maintained on the solid medium for only 35 days. A further difference in this experiment was that one of the pig inocula prepared directly from solid medium was made with very young colonies, washed off after only 3 days of incubation. Two pigs received the mycoplasma directly from solid medium and both developed typical lesions of enzootic pneumonia. One of these cases of pneumonia was passaged to two further pigs, and thence (in a second passage in pigs) to pig 2805, which also developed typical lesions (Plate 1, fig. 3). Two pigs received the mycoplasma after it had been passaged in liquid medium from solid medium; both developed enzootic pneumonia, but the lesions were more extensive than in the pigs inoculated directly from solid medium. These two cases of pneumonia were combined to make an inoculum for two further pigs, both of which developed enzootic pneumonia. Litter-mate controls were kept in all the pig passages; they received inocula prepared from the lung tissue of the appropriate previous control animals. The only control pig with lung lesions was the final one (pig 2807, which was the control for pig 2805), but these lesions were not typical of enzootic pneumonia histologically, and no pleomorphic organisms were found in the touch preparations. No significant bacteria were found in the lungs of any of the pigs in this experiment. Thus, although lesions were produced in the second main experiment by the inocula prepared directly from solid medium, once again the mycoplasma seemed more effective at inducing pneumonia after subsequent passage in liquid medium.

Recovery of Mycoplasma suipneumoniae

The pneumonia in pig 2805 was the second and final pig passage of a pneumonia induced directly with colonies from solid medium, and *M. suipneumoniae* was recovered from the lung lesions in this animal. The recovered agent was inhibited in the metabolic-inhibition test to a rabbit-serum titre of 1/320. *M. suipneumoniae* was not recovered from the control (pig 2807).

DISCUSSION

The general characteristics of the agent studied in this paper suggest that it is indeed a mycoplasma: the colonial form, the size of the elementary particles, the marked pleomorphism (with the development of filamentous forms), the requirement of a complex medium, and growth in the presence of penicillin and thallium

acetate, but not tetracycline, are all in keeping with this view. The difficulty of growing this particular mycoplasma, and the relatively slow growth of the colonies, indicate that with further work the cultural media might be improved.

The most important property of *M. suipneumoniae* is that it induces enzootic pneumonia in pigs. The mycoplasma was passaged on solid medium to a point where the final colonies which gave rise to the pig inocula were at least a 10^{-15} dilution of the first seeding onto solid medium; the induced pneumonia was then passaged serially in pigs—when it retained all the characteristics of enzootic pneumonia—and from the pneumonia in the second pig passage, a mycoplasma was recovered that was inhibited by serum prepared against *M. suipneumoniae*. It is not known, however, whether *M. suipneumoniae* is the sole cause of the disease known as enzootic pneumonia in the field. To investigate this question, we are now screening a variety of pneumonic samples obtained from outbreaks of enzootic pneumonia for strains of mycoplasma that differ serologically from *M. suipneumoniae*: it will then be necessary to see whether such strains, in pure culture, will induce a disease similar to enzootic pneumonia.

As judged by the growth-inhibition and metabolic-inhibition tests, *M. suipneumoniae* is indistinguishable from *M. hyopneumoniae* of Maré & Switzer (1965).

The comparisons with the other mycoplasmas suggest that *M. suipneumoniae* is probably a new species. We would have liked all these comparisons to have been individually complete, with all the homologous sera working well against their specific mycoplasmas. Some of the sera received from other laboratories, however, gave either a weak result or no result against their homologous culture. The simplest explanation for this is that these sera, which had often been prepared for use in other serological tests, were unsuitable for the growth-inhibition test. However, in the eight cases where the homologous serum did not inhibit growth, and in the eight cases where no homologous serum was available, none of the mycoplasmas supplied was inhibited by our sera against *M. suipneumoniae*. In all these tests, no qualitative difference was observed between the reactions obtained with the rabbit antiserum prepared against the cloned culture of *M. suipneumoniae* and the rabbit antiserum prepared against the uncloned culture. It should be pointed out that the mycoplasmas compared with *M. suipneumoniae* in the present study have not yet been compared among themselves in the literature to an extent that establishes that they are all separate species.

M. suipneumoniae was distinguishable from mycoplasma 603 in agar gel and by immuno-fluorescence, but these tests are inconvenient at the moment for routine diagnosis. It is simpler to use the growth-inhibition test for testing mycoplasma isolates from porcine respiratory disease but a problem may arise when other mycoplasmas are present concurrently; for these organisms commonly outgrow *M. suipneumoniae* in culture. The problem would be greatly simplified if a selective medium could be developed for *M. suipneumoniae*. This particular difficulty is not fully appreciated in several laboratories, where the isolation of mycoplasmas from pneumonic tissue in the pig, regardless of the pathogenicity of these organisms for pigs or their relationship to known pathogenic strains, is now being taken to indicate the presence of enzootic pneumonia.

The results obtained with the hyperimmune pig sera, using the immuno-fluorescent and double-diffusion techniques, are encouraging. We are now extending this work to include other tests, such as metabolic inhibition, working in the first instance with sera prepared against *M. suipneumoniae* in hysterectomy-produced colostrum-deprived pigs. The longer-term objective is to use sera from the field in an attempt to evaluate the role of *M. suipneumoniae* in the respiratory-disease complex of pigs.

SUMMARY

A micro-organism, previously known as the J agent, was grown on solid medium: its various characteristics suggested that it was a mycoplasma and it was provisionally named *Mycoplasma suipneumoniae.*

In the growth-inhibition and metabolic-inhibition tests, *M. suipneumoniae* was indistinguishable from *M. hyopneumoniae* (Maré & Switzer, 1965).

By the growth-inhibition test, *M. suipneumoniae* seemed unrelated to all of a wide range (42 strains) of other mycoplasmas examined. These results suggest that, if the similarity between *M. suipneumoniae* and *M. hyopneumoniae* is substantiated in further work, these latter two strains are probably a new species.

M. suipneumoniae was also identified by the metabolic-inhibition test, by precipitation in agar gel, and by immuno-fluorescence. Using the last two methods, *M. suipneumoniae* was distinguished from a second porcine mycoplasma (strain 603).

The most important property of *M. suipneumoniae* is its ability to induce enzootic pneumonia experimentally in pigs.

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EXPLANATION OF PLATE

Fig. 1. Eight-day-old growth of colonies of *Mycoplasma suipneumoniae*; these colonies were used to inoculate pigs that developed enzootic pneumonia. × 50.

Fig. 2. Culture of *Mycoplasma suipneumoniae* in liquid medium containing Hartley's broth, showing chains of cocci and globular structures of varying sizes up to 16 μ. May–Grünwald–Giesma, × 2000.

Fig. 3. Histology of pneumonia in pig 2805: final pig passage of a pneumonia induced with cultures of *Mycoplasma suipneumoniae*. Haematoxylin and eosin, × 160.

ADDENDUM

A cloned type-culture of *Mycoplasma suipneumoniae* has been deposited with Dr B. E. Andrews, Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London N.W. 9.

Fig. 1

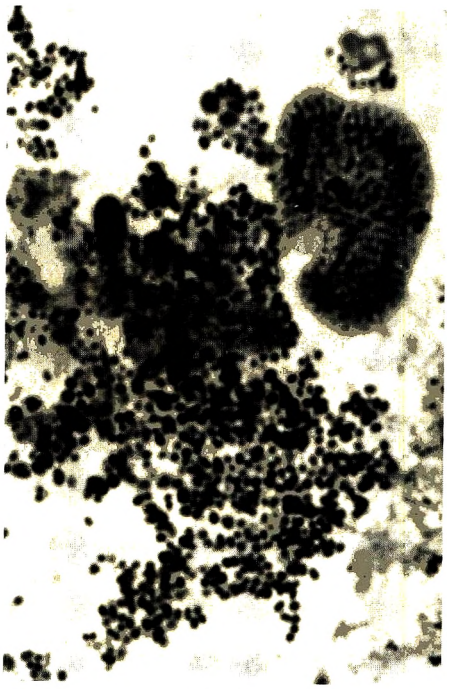

Fig. 2

Fig. 3

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Immunogenicity of experimental trachoma vaccines in baboons

III. Experiments with inactivated vaccines

By L. H. COLLIER and W. A. BLYTH
M.R.C. Trachoma Research Unit, The Lister Institute of Preventive Medicine, London, S.W. 1
and N. M. LARIN and J. TREHARNE*
Virology Department, Therapeutics Research Division, Pfizer Ltd., Sandwich, Kent

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INTRODUCTION

In preceding papers (Collier, 1961; Collier & Blyth, 1966*a*, *b*) it was demonstrated that baboons can be immunized against ophthalmic infection with trachoma/inclusion conjunctivitis (TRIC) agents by parenteral injection of live vaccines. Compared with inactivated vaccines, such preparations have disadvantages; the control of contaminants is more difficult, and unless kept at very low temperatures they rapidly lose viability, a serious drawback under field conditions. Live vaccines have been administered without ill effects to numerous baboons in the laboratory, and to some hundreds of children in pilot field trials; but the recent finding by Collier & Smith (1967) that at least one strain of TRIC agent can disseminate and multiply in primates after parenteral injection dictates caution in the further use of live trachoma vaccine, at least until more is known about the nature of such artificially induced infections. In the present paper we describe experiments with vaccines inactivated by heat, formalin and ultraviolet light, made in an attempt to avoid the disadvantages of live preparations. Experiments P 1, P 2 and P 3 were done at the Pfizer Laboratories, except that the ultraviolet irradiation in Expt P 3 was undertaken by one of us (L.H.C.) at the Lister Institute, Elstree, in collaboration with Mr L. Vallet. Experiment 11 is the last of the Trachoma Research Unit series to be described in these three papers.

MATERIALS AND METHODS

Except where otherwise stated, the materials and experimental procedures are those of Collier & Blyth (1966*a*).

TRIC agent. Strain TRIC/ /GB/MRC-4/ON (Jones, 1961; Jones & Collier, 1962), referred to for brevity as MRC-4, was used throughout. All vaccines were prepared from its 'fast-killing' variant MRC-4*f* (Reeve & Taverne, 1963); their characteristics and those of the challenge inocula are given in Table 1. MRC-4 and

* Present address: Institute of Ophthalmology, Judd Street, London, W.C. 1.

MRC-4*f* used for vaccines and challenge inocula were propagated exclusively in the chick embryo yolk sac. In the first two papers of this series, titres were expressed in terms of 50 % egg infective doses (EID 50). Here they are given as 50 % egg lethal doses (ELD 50), which for MRC-4*f* approximates closely to the EID 50, and for MRC-4 is usually half to one $\log_{10}$ unit lower.

Table 1. *Characteristics of vaccines prepared from MRC-4f and challenge inocula*

	Experiment no.				
	P 1	11	P 2	P 2 (re-challenge)	P 3
Vaccines					
Chick embryo passages ...	6	6	8	—	11
Titre ($\log_{10}$ ELD 50/ml.)	3·8	7·0*	5·8	—	5·3
Challenge inocula					
Strain	MRC-4	MRC-4	MRC-4*f*	MRC-4*f*	MRC-4*f*
Chick embryo passage	2+5 pooled	2+4 pooled	4	3	7
Titre ($\log_{10}$ ELD 50/ml.)	N.D.	3·8	N.D.	2·8	3·8

* Estimated by extrapolation; highest dilution tested killed all chick embryos.
N.D. = not done.

Table 2. *Experiment P 1: vaccination with live or heat-inactivated MRC-4f; challenge with MRC-4*

No. of baboons	Vaccine	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D. ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
8	Live	2·95	−2·67	1·04	4/8
8	Heated	5·08	−0·54	1·04	0/8
7	No vaccine	5·62	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 67; lower = 9.
L.S.D. = least significant difference.
* That is, with individual scores of 9 or less.

HEAT- AND FORMALIN-INACTIVATED VACCINES

Experiment P 1: vaccination with live or heat-inactivated MRC-4f; challenge with MRC-4

Vaccine was prepared by differential centrifugation of heavily infected yolk sacs. Part of the suspension was used as a live vaccine, and was kept at −70° C. until use. The remainder was inactivated at 37° C. for 5 days; absence of residual live TRIC agent was confirmed by two negative blind passages in chick embryo yolk sacs.

Vaccinations. Live vaccine was given to eight baboons, each of which received 1·0 ml. subcutaneously on days 0 and 6, and 1·0 ml. intravenously on day 13. An identical schedule was used for vaccinating a second group of eight baboons with inactivated suspension. Seven control animals received no vaccine.

Challenge. All animals were challenged in their right eyes on day 55, i.e. 42 days after the final intravenous dose.

Results

Whereas the live vaccines protected 4 out of 8 animals, heating at 37° C. for 5 days completely destroyed immunogenicity (Table 2).

Rechallenge after 18 *months.* The animals given live vaccine were rechallenged with a suspension of MRC-4*f* containing $10^{2\cdot8}$ ELD 50/ml.; as in previous similar experiments, they were no longer immune to conjunctival infection.

Experiment 11: *vaccination with formalin-inactivated MRC-4f; challenge with MRC-4*

Vaccine was prepared from chick embryo yolk sacs by differential centrifugation. The final suspension was treated with ultrasonic vibrations to ensure even dispersion; formalin was added to a final concentration of 0·1 % (v/v) (0·04 % formaldehyde) and the vaccine was kept at 4° C. for 46 hr., after which undiluted 0·3 ml. samples were inoculated into five chick embryos. No TRIC agent was detected in them, or in two subsequent blind yolk-sac passages. The inactivated vaccine was held at 4° C. until use; there was an interval of 34 days between the addition of formalin and the start of immunization. The titre of an untreated sample kept at 4° C. for 46 hr. was approximately 10^7 ELD 50/ml. (Table 1).

Vaccination. Six baboons each received 1·0 ml. subcutaneously on days 0 and 7, and 1·0 ml. intravenously on day 14. The six control animals received no vaccine.

Challenge with a suspension of MRC-4 (Table 1) was given 10 days after the final intravenous dose of vaccine.

Results

The mean score for the vaccinated group was somewhat higher than that for the control animals, but not significantly so; in terms of individual scores none of the vaccinated baboons gave evidence of immunity.

By contrast with live vaccines prepared from MRC-4*f* (Collier & Blyth, 1966*a*, *b*), the formalin-inactivated material induced little or no group complement-fixing (CF) antibody.

Experiment P 2: *vaccination with live or formalin-inactivated MRC-4f; challenge with MRC-4f*

Vaccine was prepared by differential centrifugation of yolk sacs infected with MRC-4*f* (Table 1); it contained 16 mg. total nitrogen per 100 ml. Half the suspension was used as live vaccine, and was stored in ampoules at −70° C. until use. To the remainder, formalin was added to give a final concentration of 0·2 % formaldehyde; inactivation was allowed to proceed at 4° C. for 5 days, during which period the suspension was twice treated with ultrasonic vibrations to facilitate penetration of formalin. Residual free formaldehyde was then titrated by a method similar to that of MacFadyen (1945); about half the quantity originally added was detectable, and was neutralized with an appropriate quantity of sodium bisulphite. The absence of live TRIC agent was confirmed by three

negative blind passages in chick embryos. The inactivated vaccine was stored at −70° C. until use.

Vaccinations. Five baboons each received 1 ml. of live vaccine subcutaneously on days 0 and 7, and 1 ml. intravenously on day 14. The same schedule was used to vaccinate six more animals with inactivated vaccine; and a third group of six baboons was given three 1·0 ml. doses of inactivated vaccine intramuscularly at weekly intervals. Seven unvaccinated baboons were used as controls.

Challenge. Seven days after the final dose of vaccine, all animals were challenged in their right eyes with MRC-4 in its third yolk-sac passage, but this inoculum failed to induce infection. Fourteen days later (21 days after the final dose) the animals were successfully challenged with a suspension of MRC-4*f* (Table 1).

Table 3. *Experiment P 2: vaccination with live or formalin-inactivated MRC-4f; challenge with MRC-4f*

No. of baboons	Vaccine	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D. ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	Live	0·40	—†	—†	4/5
6	Formalin inactivated 2 × 1·0 ml. s.c., 1 × 1·0 ml. i.v.	2·43	−1·88	1·59	2/6
6	3 × 1·0 ml. i.m.	2·91	−1·40	1·59	0/6
7	Normal yolk sac	4·31	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 47, lower = 3.
L.S.D. = Least significant difference.
* That is, with individual scores of 3 or less.
† Four animals had zero scores, which are not included in the overall analysis of variance because of statistical difficulties. However, none of the controls had zero scores, and in terms of the χ^2 test this difference between the live vaccine group and the controls is significant at about the 1 % level of probability.

Results

In terms of group mean scores, the live vaccine was more immunogenic than the formalin-treated vaccine (Table 3); and the inactivated material was rather more effective by the subcutaneous and intravenous routes than by intramuscular injection. It should, however, be mentioned that of the six animals injected intramuscularly, three had low scores (4, 4 and 5) that just failed to attain the lower confidence limit for this experiment; this was also true of the single unprotected animal in the live vaccine group, and of one of the four unprotected baboons given inactivated vaccine by the subcutaneous and intravenous routes.

The live vaccine with a titre of $10^{5{\cdot}8}$ ELD50/ml. performed noticeably better than that in Expt P 1, in which the titre was 100-fold lower.

Rechallenge after 10 months. The left eyes of all surviving animals were inoculated with the batch of MRC-4*f* used for rechallenge in Expt P 1. The upper and lower 95 % confidence limits on scores for animals rechallenged in Expt P 2 were 29 and 0. The scores for the five surviving animals originally given live vaccine were

4, 4, 1, 0 and 0. One of the two baboons originally protected by subcutaneous and intravenous injections of killed vaccine was still immune; and as might be expected, none of those given inactivated vaccine intramuscularly resisted rechallenge.

ULTRAVIOLET-INACTIVATED VACCINE

This experiment showed that ultraviolet (UV) light completely destroyed the immunogenicity of MRC-4*f*; but since there appear to be no other references to the dynamics of inactivation of TRIC agent by this method, a detailed account of the investigation is given.

Collier, McClean & Vallet (1955) described the use of the Habel & Sockrider (1947) apparatus for irradiating vaccinia virus. In brief, it consists of an inclined cylinder 5·1 cm. in diameter and 71 cm. long that rotates about its axis at 1000 r.p.m. A 24 in. Hanovia low-pressure mercury lamp is mounted axially; over 90 % of the UV emission is at 2537 Å at about 3 W. The suspension to be irradiated is introduced into the upper end of the cylinder, forms a thin film that flows past the UV source, and is collected at the lower end. Exposure can be varied by altering the flow-rate and angle of inclination, and by screening off sections of lamp to reduce its effective output. Collier *et al.* (1955) determined the amount of irradiation that completely destroyed the infectivity of purified vaccinia without impairing its antigenicity; this was assigned an arbitrary value, termed 'Relative Exposure (R.E.) 1·0'; it was twice the amount of irradiation needed to reduce the infective titre from $10^{7.5}$ pock-forming units (PFU)/ml. to $10^{0.9}$ PFU/ml.

The TRIC suspension used to vaccinate baboons was irradiated in the Habel–Sockrider apparatus; but a pilot experiment was first done by a different method to determine the degree of irradiation required in comparison with that used for vaccinia.

Comparison of inactivation rates of TRIC agent and vaccinia

TRIC agent was MRC-4*f* purified from infected yolk sacs and suspended in 0·1 M phosphate buffer, pH 7·0. It contained $10^{8.3}$ elementary bodies and 30 mg. total nitrogen per 100 ml.

Vaccinia virus was the Lister Institute strain purified from sheep lymph; the final suspension was made in 0·004 M McIlvaine buffer pH 7·2. To make its UV absorption comparable with that of the TRIC suspension it was brought to the same turbidity by adding normal yolk-sac extract.

UV irradiation was done by exposing 10 ml. samples in 9 cm. diameter Petri dishes to a Hanovia bactericidal lamp, Model 12, mounted 66·5 cm. vertically above. The characteristics of the lamp were such that a 120 sec. exposure was approximately equivalent to R.E. 1·0 in the Habel–Sockrider apparatus. Samples were rocked gently during exposure to ensure uniform irradiation. After exposure to UV for varying periods, vaccinia suspensions were titrated in the chick embryo chorioallantoic membrane (Collier, 1955); TRIC suspensions were titrated in HeLa cells (Furness, Graham & Reeve, 1960).

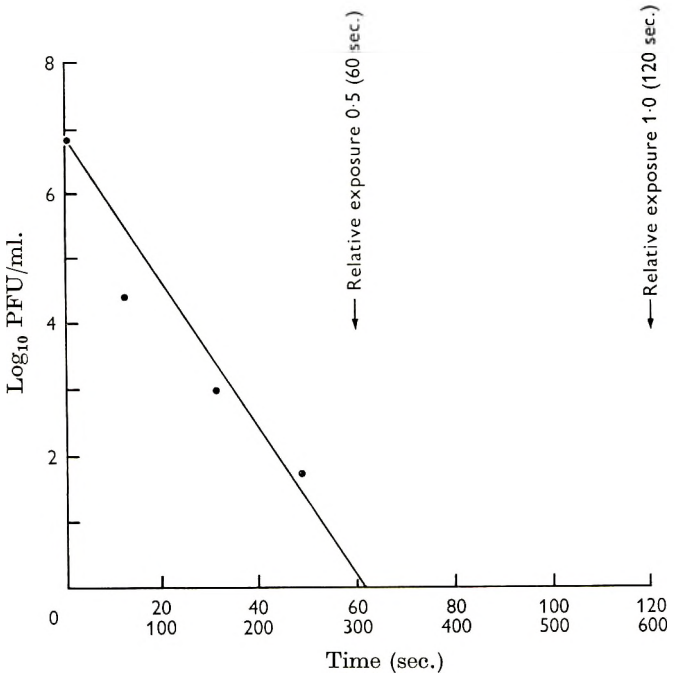

Fig. 1. Inactivation of vaccinia virus exposed in Petri dishes to ultraviolet light. The points represent titres of vaccinia virus suspension containing normal yolk-sac extract irradiated for various times on the scale 0–600 sec. The original titre was $10^{6 \cdot 8}$ pock-forming units (PFU) per ml. The solid line is derived from the data of Collier *et al.* (1955), and represents the inactivation curve of a highly purified vaccinia suspension with this starting titre. (Time-scale 0–120 sec.)

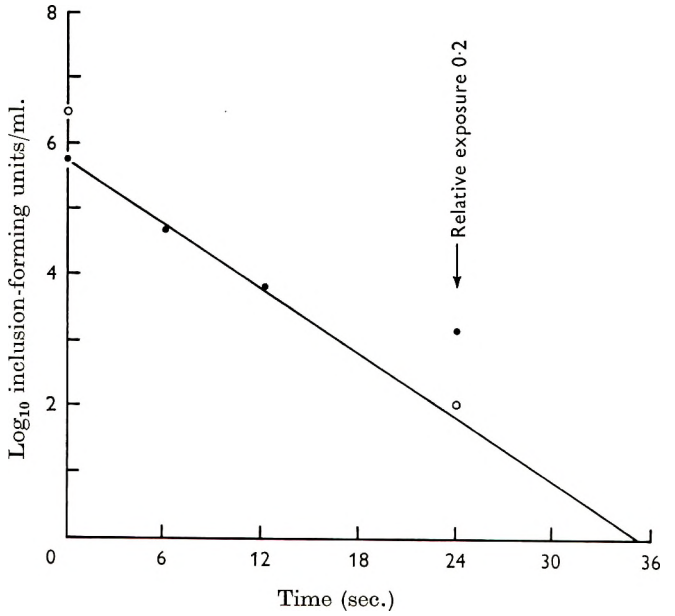

Fig. 2. Inactivation of MRC-4*f* by ultraviolet light. Closed circles, irradiated in Petri dishes. Open circles, irradiated in Habel–Sockrider apparatus.

Results

Figure 1 shows that the rate of inactivation of vaccinia virus was exponential, but in this experiment was approximately 5 times slower than that calculated from previous work with highly purified suspensions; the difference is accounted for by the presence of normal yolk-sac extract.

Figure 2 shows that inactivation of MRC-4*f* also proceeded exponentially. The unexpectedly high titre recorded after irradiation for 24 sec. was probably inaccurate because of the scanty inclusions formed on titration of this sample in HeLa cells. The rate of inactivation was calculated to be 7·5 times faster than the vaccinia suspension of comparable turbidity, and 1·5 times faster than that of highly purified vaccinia. With a TRIC suspension comparable in purity to the vaccinia preparations of Collier *et al.* (1955), the inactivation rate would presumably have been 5 (7·5 : 1·5) times as fast.

Irradiation in the Habel–Sockrider apparatus

A preliminary test in the Habel–Sockrider apparatus confirmed that MRC-4*f* was inactivated at the rate predicted from experiments in Petri dishes. A suspension with an initial titre of $10^{6·5}$ IFU/ml. was irradiated at R.E. values of 0·2, 0·4, 0·6 and 0·8. At R.E. 0·2 (corresponding to a 24 sec. exposure in Petri dishes), the titre had fallen to $10^{2·0}$ IFU/ml., and was thus close to the expected value (see Fig. 2). It was estimated that the infectivity of a suspension with this starting titre would be completely abolished at R.E. 0·3, and twice this exposure (i.e. R.E. 0·6) was chosen to inactivate the vaccine used for immunizing baboons.

Experiment P 3: *vaccination with live or ultraviolet-inactivated MRC-4f; challenge with MRC-4f*

Vaccine was prepared by differential centrifugation of yolk sacs infected with MRC-4*f* (Table 1). Part was kept as a live vaccine in ampoules at $-70°$ C. The remainder was irradiated by the Habel–Sockrider method at R.E. 0·6; this material was stored in the liquid state until use, except for a portion that was freeze-dried in 1·0 ml. amounts. Both liquid and dried suspensions were held at 4° C.

Vaccinations were done according to the schedule in Expt 11. Three groups each of six baboons were given respectively live vaccine, irradiated liquid vaccine and irradiated freeze-dried vaccine. Six control animals received a dummy vaccine made from extract of normal yolk sacs.

Challenge with MRC-4*f* (Table 1) was administered 10 days after the final intravenous dose of vaccine.

Results

Table 4 shows that live vaccine protected four out of six baboons; the scores of the two remaining animals were 1 and 4 respectively. By contrast, none of the animals given irradiated liquid vaccine was protected to a significant extent, and in only one of the group vaccinated with irradiated dried vaccine was there a significant reduction in score.

Serological findings in Experiments P 2 and P 3

The poor antibody response to formalin-treated vaccine in Expt 11 has already been noted. The serological results of the Pfizer experiments are described separately, since they were done by a method differing from that of Collier & Blyth (1966*a*), and as a routine sera were tested not at the time of challenge but at intervals before and afterwards.

Table 4. *Experiment P 3: vaccination with live or ultraviolet-inactivated MRC-4f; challenge with MRC-4f*

No. of baboons	Vaccine	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D. ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	Live	0·50	—†	—†	4/6
6	UV irradiated				
	Liquid	3·32	+0·19	1·24	0/6
	Dried	2·41	−0·72	1·24	1/6
6	No vaccine	3·13	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 33, lower = 0.
L.S.D. = least significant difference.
* That is, with individual scores of 0.
† Four animals had zero scores, which are not included in the overall analysis of variance because of statistical difficulties. However, none of the controls had zero scores, and in terms of the χ^2 test this difference between the live vaccine group and the controls is significant at about the 3 % level of probability.

Table 5. *Experiments P 2 and P 3; mean titres of complement-fixing antibody 1 week after challenge*

Expt no.	Vaccine	Mean CF titre* at time of: 1st challenge	2nd challenge
P 2	Live	36	30
	Formalin-inactivated		
	2 × 1·0 ml. s.c., 1·0 ml. i.v.	8	< 5
	3 × 1·0 ml. i.m.	17	6
	No vaccine	< 5	< 5
P 3	Live	57	—
	UV inactivated		
	Liquid	26	—
	Dried	20	—
	Normal yolk sac	13	—

* Reciprocal of geometric mean titre of antibody fixing complement with group antigen.

Complement fixation tests for antibody reacting with heated (group) antigen were done by the method of Bradstreet & Taylor (1962), using as antigen a boiled extract of yolk sacs infected with MRC-4, three minimal haemolytic doses of complement, and fixation overnight at 4° C.

Results

Table 5 gives only the results of tests made 1 week after challenge, since these are most nearly comparable with those described elsewhere in these papers. By this method of testing, the live MRC-4*f* vaccine appeared to induce lower titres than those obtained by the Trachoma Research Unit technique. In terms of group mean titres the vaccines inactivated with formalin or with ultraviolet light induced rather less antibody than did their live counterparts. The serological responses of the animals used in Expt P 1 were not determined.

DISCUSSION

The immunogenicity of a vaccine that protected baboons against conjunctival infection was destroyed at 37° C., suggesting that the protective antigen differs from the heat-stable complement-fixing antigen possessed by TRIC and related micro-organisms; this may explain why there is little or no relation between immunity and the serum titre of antibody reacting with complement and group antigen. The antigen protecting against infection seems also to differ in this respect from that which immunizes mice against TRIC toxin injected intravenously (Wang, Kenny & Grayston, 1967).

A recent review of the literature on trachoma vaccine (Collier, 1966) contains several references to the use of formalin-inactivated antigens. For example, Bietti, Guerra, Felici & Vozza (1962) maintained that this type of vaccine alone or with adjuvants protected volunteers against challenge by the conjunctival route; and Snyder *et al.* (1964) found that it significantly reduced the trachoma attack rate in Saudi Arabian children. Apart from our own experiments, however, there seem to have been no studies of the action of formalin on potency in terms of a comparison of formalin-treated preparations with equivalent doses of live vaccines. It is clear from a comparison of live and inactivated vaccines prepared from the same suspension that formalin impairs immunogenicity (Expt P 2); and although in Expt 11 the inactivated vaccine was not compared directly with a live counterpart, other experiments in this series showed that live vaccines with a similar or lower content of MRC-4*f* afforded substantial protection. In Expt P 2 the performance of the inactivated vaccine was superior to that in Expt 11 although the original titre was lower and a higher concentration of formalin was used. The technique of inactivation was, however, refined by neutralization of the residual formalin and subsequent storage at −70° C.; and it may be that prolonged exposure of the antigen to residual formalin during storage at 4° C. before use in Expt 11 accounts for this finding.

Within the limits of our observations the rate of inactivation of MRC-4*f* by ultraviolet light was exponential, and was faster than that of vaccinia virus. Experiment P 3 showed unequivocally that about twice the dose of UV light needed to abolish infectivity destroyed immunogenicity. This finding contrasts with that of Grayston *et al.* (1963) who immunized 36 Taiwanese children with a UV inactivated trachoma vaccine. The attack rate and average duration of disease were significantly less than in 53 control children, but unfortunately the

preparation and characteristics of the vaccine were not described. Collier *et al.* (1955) showed that the margin between inactivation of vaccinia and loss of antigenicity is narrow, and that the dose of UV light must in consequence be carefully controlled. Our experiment suggests that with TRIC agent the margin must be narrower still or non-existent, and it is difficult to see how the amount of irradiation used could be much reduced without the risk of failing to achieve complete destruction of infectivity.

The abolition or impairment of immunogenicity by heat, formalin and ultraviolet light may be explained in terms of damage to a protective antigen. On the other hand, live TRIC agent may be more effective as a vaccine because it multiplies after injection and thus gives rise to a greater mass of antigen than is provided by a killed suspension. The experiments reported here do not enable us to choose between these alternatives. However, Collier & Smith (1967) recently showed that MRC-4*f* did in fact multiply within baboons after parenteral injection, and that the degree of immunity resulting depended on the route of inoculation. Intravenous injection was followed by a high level of multiplication in the spleen and firm immunity to conjunctival challenge administered shortly afterwards; live TRIC agent given subcutaneously multiplied mostly in the skin and regional lymph nodes and was not so effective in inducing immunity. In the Introduction, attention was drawn to the implication of these findings for further field work with live trachoma vaccines. Our results suggest that neither heat, formalin nor ultraviolet light are satisfactory inactivating agents, and that further research on this problem is necessary.

In conclusion, it is interesting that the 'fast-killing' variant of MRC-4 used for challenge in Expts P 2 and P 3 still induced characteristic follicular conjunctivitis and inclusion bodies; another strain of inclusion conjunctivitis, TRIC/ /GB/ MRC-1/G (formerly LB 1), lost pathogenicity for the baboon conjunctiva at about the time that it acquired the ability to kill chick embryos comparatively quickly (Collier, 1962; Reeve & Taverne, 1963).

SUMMARY

Heating at 37° C. for 5 days completely abolished the capacity of a trachoma/inclusion conjunctivitis (TRIC) vaccine to protect baboons against conjunctival infection. Treatment with formalin also impaired or abolished immunogenicity.

The rate of inactivation of TRIC agent by ultraviolet light was exponential, and was about 7 times faster than that of a suspension of vaccinia virus of comparable turbidity. By contrast with vaccinia, irradiation of TRIC agent with twice the dose of ultraviolet light needed to destroy infectivity resulted in loss of immunogenicity.

The deleterious action of these inactivation procedures on the potency of TRIC vaccines may be explained in terms of damage to a protective antigen; alternatively, live vaccines may be more immunogenic because TRIC agents are capable of multiplying within primate hosts after parenteral injection.

Unlike the same type of variant of another strain of inclusion conjunctivitis, the 'fast-killing' variant of MRC-4 was still pathogenic for the baboon conjunctiva.

We are much indebted to Mr L. Vallet (Lister Institute of Preventive Medicine) for his helpful advice concerning the ultraviolet irradiation. We are most grateful to Mr P. Avis (Pfizer Ltd.) for his help with the statistical aspects of this paper.

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The first cycle of growth in the chick embryo of the agents of trachoma and inclusion blennorrhoea

By P. REEVE and JANICE TAVERNE
M.R.C. Trachoma Research Unit, The Lister Institute of Preventive Medicine, London, S.W. 1

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We have observed differences in the rate of growth of strains of trachoma and inclusion blennorrhoea agent in the chick embryo consistent with the assumption that increased virulence for the chick embryo is associated with a higher rate of multiplication (Taverne, Blyth & Reeve, 1964). Growth curves were plotted from the time of inoculation to the death of the embryo by daily measuring the amount of agent formed in terms of lethality for chick embryos, infectivity for HeLa cells and total particles; these curves represented the results of many cycles of multiplication. However, the differences in growth rate of strains of different virulence were small and the data were not amenable to tests of statistical significance; furthermore, the finding of such differences was at variance with earlier deductions from dose–response curves plotted from the results of egg titrations (Jawetz & Hanna, 1960; Reeve & Taverne, 1963).

We pointed out that differences in growth rate over a long period do not necessarily reflect differences of intracellular growth (Taverne *et al.* 1964), but could, for instance, result from variations in behaviour during the extracellular phase. To distinguish between these possibilities and to verify differences in growth rate, single-cycle growth curves were plotted for several strains of trachoma and inclusion blennorrhoea agents multiplying in the chick embryo yolk sac; we have compared their intracellular cycles of growth and related them to the growth rates previously observed.

MATERIALS AND METHODS

TRIC agents listed below are designated according to the system proposed by Gear, Gordon, Jones & Bell (1963). Abbreviated designations are used in the text (Collier, 1963).

For convenience, *f* is suffixed to the designation of strains that kill chick embryos relatively quickly ('fast-killing variants': Taverne *et al.* 1964) by contrast with the less virulent, slow-killing strains for which the suffix *s* is used where necessary for clarity.

Strains. TRIC/ /China/Peking-2/OT (T'ang, Chang, Huang & Wang, 1957) was obtained from freeze-dried material preserved by I.C.I. Laboratories, Cheshire.

TRIC/ /SAU/HAR-2/OT (Murray *et al.* 1960) (SA 2). The variant, HAR-2*f* was derived at the Lister Institute from material supplied by Dr S. Bell, School of Public Health, Harvard University.

TRIC/ /GB/MRC-4/ON (Jones, 1961) (LB 4) was isolated from material supplied by Professor Barrie Jones, Institute of Ophthalmology, London. The variant LB 4*f* was derived at the Lister Institute.

TRIC/ /WAG/MRC-1/OT (Collier & Sowa, 1958) (G 1) was isolated in the Gambia and maintained at the Lister Institute.

Diluent was phosphate-buffered saline containing streptomycin sulphate, 1000 μg./ml.

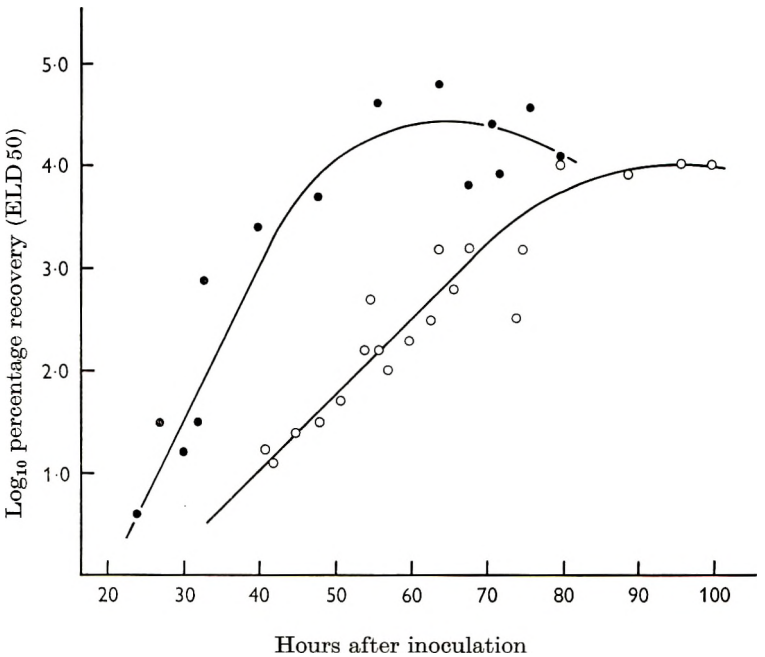

Fig. 1. The first cycle of growth in the chick embryo yolk sac of strains of trachoma and inclusion blennorrhoea of differing virulence. The points represent geometric means ($\log_{10}$ ELD50 per YS) expressed as a percentage of the dose inoculated. ●, Mean of strains HAR-2*f* and MRC-4*f*; ○, mean of strains MRC-1/OT, MRC-4 and PK-2.

Infectivity titrations were done in the yolk sacs of 7-day chick embryos kept at a continuously recorded temperature of 35° C. ± 0·5° C. in a humid atmosphere. Eggs were candled daily and tests were terminated 14 days after inoculation. Titres were calculated according to the method of Reed & Muench (1938) and expressed in terms of 50 % lethal doses for eggs (ELD50). Specificity of death, when doubtful, was determined by examining yolk-sac smears for the presence of elementary bodies.

Growth curves. For each strain, the yolk sacs of fifty 7-day chick embryos were inoculated with 0·5 ml. of a yolk-sac suspension calculated to contain between 10 and 100 ELD50/ml. and titrated at the time of injection. At intervals of about 8 hr. the yolk sacs of 3 or 6 live embryos were harvested, shaken with glass beads in 10 ml. of diluent, and titrated in eggs using 3·2-fold serial dilutions.

Results were expressed as ELD 50 recovered per yolk sac, and in Fig. 1 as a percentage of the inoculum, i.e.

$$\frac{\text{ELD 50 inoculated per yolk sac}}{\text{ELD 50 recovered per yolk sac}} \times 100.$$

RESULTS

The amount of agent present in yolk sacs at different times during the first 3 days after inoculation of fast- and slow-killing strains was measured in ELD 50 per yolk sac (Table 1). Growth curves were plotted to show the increase in infectious organisms during this period by calculating the geometric means for the two *f* and three *s* strains respectively, and expressing them as a percentage of the mean dose inoculated for each group (Fig. 1).

With strains HAR-2*f* and MRC-4*f*, infectious organisms were detected 24 hr. after inoculation and thereafter their number increased exponentially to reach a peak about 60 hr. after inoculation. After this time the amount of agent decreased, indicating that one cycle of multiplication was complete. With strains MRC-4, MRC-1/OT and PK-2, organisms were first detected about 40 hr. after infection and their number increased exponentially until about 80 hr.

These results were analysed to determine any statistically significant differences in the behaviour of *f* and *s* strains in terms of (*a*) the times when exponential growth began and (*b*) the slopes of the exponential sections of their growth curves. Exponential growth was taken as starting when the amount of agent recovered per yolk sac, expressed as a percentage of the dose inoculated, was 1 ELD 50 by extrapolation; this time was on average 21 hr. after inoculation for the *f* strains and 38 hr. for the *s* strains. The difference between these times was significant at the 1% level ($t = 3{\cdot}19$ with 28 D.F.; $0{\cdot}01 < P < 0{\cdot}002$). If the exponential phase is taken as ending at 40 hr. with *f* strains and at 68 hr. with *s* strains, comparison of the slopes suggested a genuine difference which just fails to attain significance at the 5% level of probability ($t = 2{\cdot}03$, with 28 D.F.; for $t = 2{\cdot}05$, $P = 0{\cdot}05$). Estimates of the average slope of the growth curves gave growth rates of $10^{0{\cdot}14}$ ELD 50 per hr. for the *f* strains and $10^{0{\cdot}07}$ ELD 50 per hr. for the *s* strains.

DISCUSSION

The growth rate of an organism can be measured directly as the rate of division during the exponential phase of one cycle of growth, or it can be calculated from the yield in unit time. A rate derived in the first way is defined here as the 'actual growth rate', as it measures the true rate of division. The second method gives an 'apparent growth rate' since during the observation period there may be a lag phase in which the organism is not actively dividing, and some organisms may die before samples are titrated. Previously, we found that during several cycles of growth the more virulent strains had faster apparent rates of growth than the less virulent (Taverne *et al.* 1964). Measurement of the maximum yield obtained in unit time after one cycle of growth confirms the existence of these differences in apparent growth rate related to differences in virulence. Thus, calculated from

Table 1. *Yields of TRIC agents, in log_{10} ELD50 per yolk sac during the first cycle of growth*

Strain ...	HAR-2*f*			MRC-4*f*	MRC-1/OT		MRC-4					PK-2		
Suspension* ...	*a*	*a*	*b*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*b*	*c*
Dose inoculated ...	1·7	2·6	2·5	1·2	2·0	1·7	1·8	3·6	3·0	2·3	2·5	2·5	1·9	3·6
Hours after inoculation														
24	0·6	—	1·9	< 0·0	< 0·0	—	1·2	0·7	—	—	—	< 0·0	—	—
27	—	—	2·0	—	—	—	—	—	—	—	—	—	—	—
30	—	—	1·7	—	—	—	—	—	—	—	—	—	—	—
32	1·1	—	—	0·8	< 0·0	—	< 0·0	2·2	—	—	—	< 0·0	—	—
33	—	—	3·4	2·9	—	—	—	—	—	—	—	—	—	—
36	—	—	—	—	—	—	—	—	< 0·5	—	—	—	—	—
40	2·7	—	—	—	—	—	< 0·0	—	—	—	—	—	—	—
41	—	—	—	—	1·9	—	—	2·8	—	—	—	1·1	—	—
42	—	—	—	—	—	—	—	—	1·4	—	—	—	—	—
45	—	—	—	—	—	—	—	—	—	—	1·9	—	—	3·3
48	3·5	—	—	2·8	1·1	—	2·6	3·7	3·1	—	2·4	1·6	2·0	2·0
51	—	—	—	—	—	—	—	—	—	—	2·5	—	—	3·0
54	—	—	—	—	—	—	—	—	—	2·1	3·1	—	—	3·8
55	—	—	—	—	—	—	—	—	—	—	—	—	3·0	—
56	3·7	4·3	—	4·5	2·2	2·4	1·8	3·6	—	—	—	2·7	—	—
57	—	—	—	—	—	—	—	—	—	2·4	2·4	—	—	3·5
60	—	—	—	—	—	—	—	—	—	—	3·1	—	—	3·6
63	—	—	—	—	—	—	—	—	—	2·8	3·0	—	—	4·0
64	3·9	4·8	—	4·5	—	4·0	1·7	—	—	—	—	—	—	—
65	—	—	—	—	—	—	—	—	—	—	—	3·5	3·6	—
66	—	—	—	—	2·5	—	—	4·6	—	—	—	—	—	—
68	—	4·2	—	—	—	2·9	—	—	—	—	—	—	—	—
71	—	—	—	3·6	—	—	—	—	—	—	—	—	—	—
72	3·7	4·5	—	—	2·1	—	2·5	4·0	—	—	—	2·4	2·9	—
74	—	—	—	—	—	—	—	—	—	—	—	—	—	—
75	—	—	—	—	—	3·6	—	—	—	—	—	—	—	—
76	—	4·9	—	—	—	—	—	—	—	—	—	—	—	—
78	—	—	—	—	—	—	—	—	—	—	—	—	2·4	—
80	—	4·7	—	—	—	3·7	—	—	—	—	—	—	—	—
89	—	6·4	—	—	—	3·9	—	—	—	—	—	—	3·4	—
96	—	7·6	—	—	—	3·7	—	—	—	—	—	—	—	—
101	—	—	—	—	—	—	—	—	—	—	—	—	3·9	—
105	—	—	—	—	—	4·5	—	—	—	—	—	—	—	—

* Identity of pool used as inoculum.
A dash indicates that a sample was not taken at that time

Fig. 1, the apparent growth rate of *f* strains was $10^{1.9}$ ELD50 per day and of *s* strains was $10^{1.1}$ ELD50 per day.

Measurement of the slopes of the exponential phases of single-cycle growth curves strongly suggested concomitant differences in actual growth rate. The more virulent strains MRC-4*f* and HAR-2*f* multiplied at a rate of $10^{3.4}$ ELD50 per day, compared with a rate of $10^{1.7}$ ELD50 per day for the less virulent strains MRC-1/OT, MRC-4 and PK-2.

The significance of other differences observed in the growth cycle of the two sorts of strain is not clear. The observed difference in the length of their lag phase may not be real; the less virulent strains require 10–100 times more elementary bodies to form an ELD50 than do *f* strains (Reeve & Taverne, 1963; Taverne *et al.* 1964) so that there may be a period during which elementary bodies of *s* strains are increasing in number but are not sufficiently numerous to be detected by titration in eggs. The techniques available for counting elementary bodies are not sensitive enough to settle this question.

This study has confirmed statistically that the greater virulence of some TRIC strains for the chick embryo is associated with a faster growth rate. The fundamental difference between the strains lies in their particle/infectivity ratios and its basis will not be resolved until a method is devised for accurately enumerating those *s* strain elementary bodies that cannot be detected in eggs.

SUMMARY

Chick embryos were inoculated with measured doses of various strains of the agents of trachoma and inclusion blennorrhoea, and the number of infective organisms they contained was determined at intervals during the first 4 days after inoculation. From curves of the yield of agent at different times relative to the dose inoculated, it was evident that the lag phase before the exponential phase of growth began was shorter for fast-killing—and more virulent—variant strains than for slow-killing strains, and the difference was statistically significant; and that variant strains multiplied faster during the exponential phase of growth and produced their highest titres sooner.

We wish to thank Dr Ian Sutherland of the M.R.C. Statistical Research Unit for his statistical analysis of our results. We are grateful to Prof. L. H. Collier for help with our manuscript and to Miss Christina Payne for her invaluable technical help.

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Experiments on terminal disinfection of cubicles with formaldehyde

By M. M. BEEBY, D. KINGSTON and C. E. WHITEHOUSE
Cross-Infection and Disinfection Reference Laboratories, Central Public Health Laboratory, Colindale Avenue, London N.W. 9

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The report of the Committee on Formaldehyde Disinfection (Public Health Laboratory Service, 1958*a*) dealt mainly with the use of formaldehyde for sterilizing contaminated fabrics such as bedding. Their results, and the practical experience at the Microbiological Research Establishment, Porton, formed the basis for the fumigation procedures recommended in the note on the Practical Aspects of Formaldehyde Fumigation (Public Health Laboratory Service, 1958*b*). The work here reported was undertaken to explore further the advantages and limitations of this procedure in disinfecting isolation cubicles in hospitals.

The test specimens were prepared from *Staphylococcus aureus* and *Pseudomonas aeruginosa*, representing two important and very different pathogens. Dust was used to represent comminuted contamination into which the formaldehyde would readily penetrate. Dried broth drops represented substantial smears of contamination into which penetration might be more difficult. Really massive contamination was not represented as this should be removed mechanically. In addition, in some experiments the spore test piece of Beeby & Whitehouse (1965), suggested for the control of ethylene oxide sterilization, was included to see if it could serve as a control for formaldehyde fumigation.

The test specimens were exposed to formaldehyde vapour for 3 or 24 hr. under controlled conditions in the laboratory or in rooms into which formaldehyde had been liberated. Viable counts were carried out and the survivals after exposure compared with those in control specimens. In addition, measurements were carried out to determine the formaldehyde concentrations and humidities at different times.

MATERIALS AND METHODS

Media

Broth. Oxoid nutrient broth no. 2.

Serum. Horse serum no. 2. (Burroughs Wellcome and Co.).

Elution fluid. Broth or, for the spore test pieces, quarter-strength Ringer's solution with 0·1 % (v/v) Tween 80 (Honeywill-Atlas Ltd.).

Diluent. Quarter-strength Ringer's solution or broth-saline (physiological saline with 5 % (v/v) broth added).

Nutrient agar. Broth solidified with 1·2 % (w/v) Oxoid agar no. 3.

Serum agar. Broth solidified with 1·1 % (w/v) Davis New Zealand powdered agar with the addition of 5 % (v/v) horse serum before pouring.

Test contamination

Organisms

Staph. aureus NCTC 6571 ('Oxford'), *Ps. aeruginosa* NCTC 6749 and *Bacillus subtilis* NCTC 10073 (Camp Detrick strain) were used. Owing to the erratic survival of the *Ps. aeruginosa* it was used only in a limited number of experiments. The organisms were grown at 37° C.

Preparation

Dust. This was prepared by a procedure similar to that used by Kingston & Noble (1964). Two serum agar plates were flooded with a broth culture of *Staph. aureus* and incubated overnight. The surface growth was rubbed off in 10 ml. of broth and this suspension mixed into 10 g. of cotton dust. In some experiments the growth was suspended in horse serum. The impregnated dust was dried *in vacuo* over $CaCl_2$ for 7 hr. and allowed to stand over saturated $Na_2Cr_2O_7$ solution overnight to bring the water content towards equilibrium with a relative humidity of 52 %. The dust was then ground in a Waring blender. The prepared dust gave counts of the order of 10^9 organisms per g. (for detailed figures see Table 5, where the counts are given per 0·2 g. specimen, or per 0·1 g. for the 'bijou' specimens).

Dried drops. The suspensions of *Staph. aureus* were overnight broth cultures. For *Ps. aeruginosa* the surface growth on a nutrient agar slope after overnight incubation was washed off into 20 ml. of broth, and the resulting suspension was shaken by hand with glass beads for 1 min. to help break up the clumps. To prepare suspensions of the organisms in serum, the *Staph. aureus* culture was spun down and resuspended in about an equal volume of serum; the *Ps. aeruginosa* growth was washed off in it.

Strips of high-density polythene (4·1 × 1·6 × 0·008 cm.) were boiled for 5 min. in distilled water and allowed to dry. Each was inoculated with a drop (*ca.* 0·02 ml.) of the appropriate suspension. The drops were allowed to dry overnight over saturated $Na_2Cr_2O_7$ (52 % relative humidity). The prepared strips usually had counts of about 10^6–10^7 organisms (for detailed figures see Tables 1 and 5).

Spore test pieces. These were prepared by the method of Beeby & Whitehouse (1965). One drop (*ca.* 0·02 ml.) of a suspension of spores in 90 % (v/v) methanol in water was dropped on a piece of clean sterile aluminium foil (2 × 1 cm.). The suspension was used in two concentrations, containing respectively about 10^6 and about 10^3 spores per drop.

Exposure to formaldehyde

Desiccator experiments

The base of each desiccator contained about 150 ml. of an appropriate saturated salt solution with excess solid to control the humidity. The salts used and the corresponding relative humidities at 20° C. were as follows (O'Brien, 1948):

Table 1. *Survival of organisms in dried drops and of spore foils when exposed to formaldehyde in desiccators*

Each survival is given as a percentage of the count at the start of the experiment and, except as below, is derived from the mean of counts on 3 strips. The control survivals are derived from strips exposed at the same humidity in the absence of formaldehyde.

Relative humidity (%)	Formaldehyde (mg./l.)	Organism	Vehicle	No. of organisms per strip*		Percentage survival after exposure			
						Test strips		Controls	
				Thousands	% of inoculum	3 hr.	24 hr.	3 hr.	24 hr.
86	0·9	*Staph. aureus*	Broth	5000	83	0†	0	49	49
			Serum	2000	34	0	0	80	58
		Ps. aeruginosa	Broth	3000	5·0	0	0	33	⩽ 0·009‡
			Serum	200	3·8	0	0	⩽ 0·1	⩽ 0·1
		B. subtilis	—	400	N.D.§	⩽ 0·07	0	42	45
86	0·4	*Staph. aureus*	Broth	2000	12	0	0	13	87
			Serum	1000	17	⩽ 0·03	⩽ 0·03	240	22
		Ps. aeruginosa	Broth	20	0·055	⩽ 2	0	260	6·3
			Serum	10	0·13	⩽ 2	0	0·24	⩽ 2
		B. subtilis	—	500	N.D.	2	0	62	80
			—	0·3	18	90	0	81	170
58	0·6	*Staph. aureus*	Broth	3000	59	0·083	0·031	32	8·4
			Serum	3000	44	27	⩽ 0·01	56	14
		B. subtilis	—	0·3	N.D.	130	⩽ 9	170	120
32	0·7	*Staph. aureus*	Broth	3000	59	66	17	35	14
			Serum	3000	44	51	39	49	21
		B. subtilis	—	0·3	N.D.	89	0	160	120

* The number per strip is given in thousands and is the mean count of three strips estimated at the start of the experiment in the same way as the other survivals. It is also given as the percentage of the number of organisms per drop of the suspension used to prepare the strips. This percentage thus shows the loss in drying, etc., and the loss (if any) by adhesion to the strip.

† A zero indicates that there was no growth of the appropriate organism on the agar plates or when the strips were incubated with the remains of the eluate.

‡ ⩽ 0·009, etc., indicates survival equal to or less than the stated figure. There were no colonies formed from any of the dilutions plated, but at least one of the three strips incubated with the remains of the eluate showed growth of the appropriate organism. The stated figure corresponds to 3 colonies at the lowest dilution.

§ Not done.

KCl, 86%; NaBr, 58%; $CaCl_2$, 32%. Sufficient formaldehyde solution had been added to give the vapour concentration required.

The specimens, which were dried drops and spore test pieces only, were laid out on Petri dishes in a way that allowed free gas exchange with the saturated salt solution. Control specimens were exposed in identical desiccators to saturated salt solutions without formaldehyde. The desiccators were kept on the bench out of direct sunlight.

Room experiments

Dust was exposed in 0·2 g. lots in open unguent jars (5 cm. diam., 3 cm. deep), dispersed as evenly as possible over the base of each jar. Also, as a model for contamination to which the formaldehyde could penetrate only by diffusion, 0·1 g. of dust was placed in each of a number of $\frac{1}{2}$ oz. screw-capped ('bijou') bottles which had a 0·5 cm. diameter hole drilled through both cap and rubber liner, and four thicknesses of cotton gauze between the two. This test object was modified from that used by Dr R. M. Fry (personal communication), who used blanket in place of the gauze. Dried drops of broth were exposed by leaving the inoculated polythene strips in open Petri dishes, and spore test pieces were exposed similarly.

Cubicles at West Hendon Isolation Hospital with a volume of about 1500 cu.ft. were used. These had walls and ceilings covered with glossy paint, and floors of unpolished wood boards. Each contained an iron bedstead and a wooden locker, but no mattresses or curtains. One experiment was carried out in a room at the Central Public Health Laboratory with a volume of about 2000 cu.ft. The walls and ceilings were covered with glossy paint and the floor was of polished composition tile. There were ordinary laboratory benches of waxed teak. In preparing the rooms for fumigation the ventilators were closed and the cracks round the windows sealed with Sellotape. As soon as fumigation had been started the doors were also sealed with Sellotape.

Formaldehyde was vaporized by the reaction of formalin with potassium permanganate in the quantities recommended by the Public Health Laboratory Service (1958*b*). For the 1500 cu.ft. isolation cubicles, 255 g. of $KMnO_4$ were added to 750 ml. of formalin (40%, w/v, formaldehyde) in a small aluminium chamber pot standing in a large enamel basin in the middle of the floor. The reaction resulted in some spilling into the basin and the nearly complete evaporation of all liquid. Twice the recommended amount was used in the laboratory room.

In some experiments on the concentrations of formaldehyde and water vapour attained in the cubicles, formaldehyde solutions were boiled off from various electrically heated vessels.

Estimation of survival

Dust samples

These were shaken up with 10 ml. of nutrient broth and the number of organisms in the broth was estimated by inoculating suitable dilutions on the surface of serum agar plates. Colonies were counted after at least 24 hr. incubation at 37° C.

The mixture of broth and dust was not incubated, because it sometimes contained sufficient formaldehyde to inhibit bacterial growth.

Dried drops

The polythene strips were shaken up with 10 ml. of nutrient broth (control specimens) or 5 ml. (test specimens) and survival estimated in the same way as for the dust. In addition, the strips were incubated in the remains of the broth since there was not enough formaldehyde carried over to cause trouble. If the broth showed growth this was recorded as positive only if subculture showed the organism to be the test organism.

Spore test pieces

Each of these was shaken with glass beads in 10 ml. of quarter-strength Ringer's solution to which 0·1 % (v/v) Tween 80 had been added. Tenfold dilutions were inoculated on the surface of serum agar plates. An equal quantity of double-strength broth was added to 5 ml. of the elution fluid and this was incubated with the test piece for 2 days.

Carry-over of formaldehyde

Possible danger from this was investigated in the following experiments. Desiccators were prepared with relative humidities of 86 %, 58 % and 32 %, and formaldehyde vapour concentrations of 0·9 mg./l. (see section on Exposure to formaldehyde). Prepared strips with broth or serum drops were exposed in these for 2 days, removed, and inserted into screw-capped bottles containing 5 ml. of broth (cf. section on Estimation of survival). Each bottle was then inoculated with one drop of a suspension of *Staph. aureus* or of *Ps. aeruginosa*, containing 18 and 28 organisms respectively. Growth occurred with both organisms in all the bottles containing single strips—that is, under the conditions used for estimating survival. When pairs of strips were inserted growth still occurred, with the sole exception that pairs with serum drops after exposure to 86 % relative humidity inhibited both organisms. Under the same conditions pairs with broth drops did not inhibit. Owing to the uptake of water vapour, more formaldehyde will be carried over at high humidities, and these provide the most critical situation. It was thought therefore that as far as the strips were concerned there was unlikely to be trouble from the carry-over of formaldehyde.

In a further experiment, 0·2 g. samples of dust prepared from broth or serum suspensions were exposed similarly to formaldehyde. These were suspended in 5 ml. of broth—that is, half the amount used in estimating survival—and the suspensions inoculated with *Staph. aureus* and *Ps. aeruginosa*. The correct number of colonies grew when 0·1 ml. portions of this broth were spread without dilution on to serum agar plates, though no growth occurred in the broth itself. When 0·4 g. of dust was used the number of colonies of *Ps. aeruginosa* developing was reduced to about two-thirds, though there was no significant reduction for *Staph. aureus*. Since therefore signs of inhibition only started to appear with a fourfold concentration (twice the quantity of dust in half the volume of elution fluid), it was thought that there was an adequate margin of safety.

Estimation of formaldehyde

Twenty-five ml. of air was aspirated through a narrow piece of plastic tubing into a 30 ml. syringe containing 5 ml. of distilled water. The syringe was shaken vigorously for about $\frac{1}{2}$ min. before the water was expelled into a screw-capped bottle (cf. Public Health Laboratory Service, 1958*a*). The formaldehyde content was estimated colorimetrically by the method of Nash (1953) within 5 hr. of the collection of the sample. It was shown that keeping the solutions on the bench for up to 24 hr. did not affect the formaldehyde estimation.

In sampling air from the cubicles about 2 ft. of the tubing was run between the door and the frame, between the upper edge and the lintel, the end being about 2 in. away from the door. In sampling from the desiccators about 9 in. of tubing was used, run in through the stopcock. The tubes were washed through with the air to be estimated before the sample was taken. The concentrations in the desiccators are the mean of determinations carried out just before removing the lid on at least two of the following occasions: before inserting the specimens, before taking the 3 hr. and before taking the 24 hr. sample. All samples were taken and estimated in duplicate.

Estimation of relative humidity

In the desiccator experiments, the relative humidity was assumed to be that in equilibrium with the saturated salt solution. In the cubicle experiments, the determinations before vaporization were made with a whirling hygrometer and subsequent determinations were made with an aspirating (Assmann) hygrometer which drew the air for the wet and dry bulbs through two rubber tubes run between the door and the lintel.

RESULTS

Desiccator experiments

The organisms were exposed on polythene strips in dried drops of serum or broth, with the exception of the spore test pieces (Table 1). At 86% relative humidity *Staph. aureus* and *Ps. aeruginosa* were satisfactorily killed by formaldehyde. With a concentration of 0·9 mg./l. there was no growth of the test organism from any of the strips after 3 hr. With a concentration of 0·4 mg./l. kill was not quite complete. At 32% relative humidity the formaldehyde had little significant effect on *Staph. aureus* or on *B. subtilis* spores. At 58% relative humidity there was a substantial kill, but this was never complete, all strips still showing growth after 24 hr. exposure. The spore test strips were included to see if they could be used to control formaldehyde fumigation. The suggestion from these experiments is that the disinfectant action of formaldehyde may be less dependent on humidity for the spores than it is for the other test strips. After 24 hr. all the spore strips at all the humidities failed to show growth, with the exception of 2 out of 3 at 58% relative humidity. With the exception of the highest humidity and formaldehyde concentration, all the spore strips survived fairly well after 3 hr.

Table 2. *Formaldehyde and water-vapour concentrations after vaporizing formalin in rooms*

(Expts 1–9: in isolation cubicles (vol. 1500 cu.ft.). Expt 10: in a room (vol. 2000 cu.ft.). Expts 1–5 and 10: vaporization by the addition of permanganate to formalin (occurred rapidly). Expts 6–9: formalin vaporized electrically. The time taken to vaporize was ½ hr., 1 hr., 1½ hr. and 2 hr. respectively, and the 0 min. samples were taken immediately after this.)

Expt no.	40% formalin (ml.)	Extra water (ml.)	Relative humidity (%)						Formaldehyde concentration (mg./l. air). Time after vaporization:						
			Before vaporization	Time after vaporization: 0 min.	5 min.	15 min.	3 hr.	24 hr.	0 min.	5 min.	20 min.	30 min.	1½ hr.	3 hr.	24 hr.
1	750	—	26	—	—	52	36	—	—	—	1·9	1·6	—	0·3	—
2	750	—	70	—	83	—	64	—	—	0·9	—	—	—	1·2	—
3	750	—	57	—	86	—	64	—	—	1·6	—	—	—	1·0	—
4	750	—	65	—	86	—	—	56	—	1·4	—	—	—	—	0·1
5	750	—	55	—	82	—	—	56	—	1·7	—	—	—	—	0·3
6	375	750	54	83	—	—	—	45	0·6	—	—	—	—	—	0·05
7	750	1500	54	90	—	—	—	50	0·9	—	—	—	—	—	0·2
8*	750	1500	50	89	—	—	—	45	1·0	—	—	—	—	—	0·08
9	?†	?†	51	91	—	—	—	50	0·7	—	—	—	—	—	0·05
10*	2000	—	55	—	—	75	—	—	1·9	—	—	—	1·1	0·9	—

* A fan was run in the room or cubicle throughout the experiment.

† Vaporization (of 1500 ml. of formalin and 3 l. of water) was not complete, and the amounts actually vaporized are not known, since the proportions of formaldehyde and water in the residuum were not determined.

Cubicle experiments

Formaldehyde and water-vapour concentrations

It is not possible to predict the concentrations of formaldehyde and water vapour that will be achieved when formalin is vaporized in a room. A number of experimental determinations were therefore made and these are set out in Table 2. We discuss first the experiments (1–5) in which the formalin was vaporized by reaction with $KMnO_4$ in the quantities suggested by the Public Health Laboratory Service (1958*b*). The results show that the increase in the percentage relative humidity was never more than 25, and sometimes less. The 3 hr. determinations suggest that the humidity falls fairly rapidly. The formaldehyde concentrations remained adequate up to 3 hr. after vaporization (Expts 2 and 3). After 24 hr. only traces were left, probably below the level at which estimation was likely to be accurate (Expts 4 and 5). In Expt 1 the sealing of the cubicle was known to be unsatisfactory, and the concentration of formaldehyde fell rapidly.

Table 3. *Concentration of formaldehyde at top and bottom of door*

Experiment no.	Time of sample (hr.)	Concentration of formaldehyde (mg./l. air)	
		Top of door	Bottom of door
6 (no fan)	0	0·8, 0·7	0·4, 0·6
7 (no fan)	0	0·9, 1·0	0·8, 0·7
9 (no fan)	0	0·8, 0·8	0·5, 0·5
8 (fan)	0	1·2, 0·9	1·1, 0·9
10 (fan)	0	2·0, 1·7	1·8, 1·9
	1	1·4, 1·5	1·1, 1·4
	1½	1·2, 1·2	1·1, 0·9
	2½	0·8, 0·9	0·9, 0·9
	3	0·8	1·0

Experiment 10 was carried out in a room at the Central Public Health Laboratory with twice the suggested quantities of formalin and permanganate. The humidity rise was still only 20. The formaldehyde concentration was higher initially, but fell more rapidly, probably because pressure difference within the large building made the sealing less effective.

The results found when formalin solutions were boiled off electrically (Expts 6, 7, 8 and 9) cannot be compared exactly with those previously discussed, since vaporization by electrical heating took much longer. The water-vapour concentrations were much more satisfactory. Except in Expt 6 in which half quantities were vaporized, there was considerable fogging and condensation on the walls and window, showing that the air was saturated. The initial humidity measurements of around 90 % are therefore misleadingly low, probably owing to temperature variations. The formaldehyde concentrations of about 1 mg./l. are satisfactory, particularly as these levels are likely to have existed during vaporization. They were, however, rather low when half quantities were used or vaporization was not complete (Expts 6 and 9).

A check was made on the uniformity within the rooms by estimating the formaldehyde concentrations near the top and near the bottom of the doors, with and without a fan being run. Such determinations were carried out in Expts 6–10. The concentrations after 24 hr. were too low to give results of sufficient accuracy, but the other results are set out in Table 3. It can be seen that without a fan the concentrations found near the floor were lower, whereas no consistent differences were found when a fan was run. The differences were not, however, very large. Estimations of this sort are probably only capable of measuring concentration differences in the main body of the air in a room. The layer of comparatively still air next to a surface, the 'boundary layer', may have a concentration appreciably different to that in the bulk of the room air if the surface is absorbing or evolving formaldehyde, and it is in the boundary layer that the bacteria are to be found.

The interpretation of the formaldehyde concentrations found presents some difficulty. The maximum vapour-phase concentration of formaldehyde at 20° C. is about 2 mg. per litre of air, limited by the tendency of formaldehyde to polymerize to polyoxymethylene derivatives. (For a general account of the chemistry of formaldehyde see Walker (1964). The tables on pp. 113 and 150 summarize the data on the vapour pressures of formaldehyde over formalin and over paraformaldehyde respectively.)

The quantity of formalin suggested, 500 ml. per 1000 cu.ft., corresponds to 7·1 mg. of formaldehyde per litre of air. In fact a concentration of 2 mg./l. only was achieved in Expt 10 where twice the suggested quantities were used. Oxidation by the permanganate cannot be the explanation since, if this reaction follows the stoichiometric equation $4KMnO_4 + 3H_2CO = 4KOH + 4MnO_2 + 3CO_2 + H_2O$, 170 g. of $KMnO_4$ could oxidize only 24 g. of formaldehyde. Since there is always some air exchange through cracks in the floor boards, etc., ventilation might remove a substantial amount of formaldehyde during the comparatively slow vaporization from the electrically heated containers. If there were a steady concentration of 2 mg./l. and the improbably high ventilation rate of 1 air change/hr., 113 g. of formaldehyde would be lost. This is not quite enough. However this process would not explain the low concentrations found after the very rapid vaporization with $KMnO_4$. It seems probable the explanation is adsorption of formaldehyde on to the walls and furniture. This is known to occur (Harry, 1954). Adsorption would be reversible and give a buffering action against the removal of formaldehyde by ventilation. The small drop of formaldehyde concentration over 3 hr., which would correspond to an improbably low ventilation rate of about 0·1 air change/hr., suggest that this occurred. It was also found by Harry (1954). Such a buffering effect would also be produced by vaporizing sufficient formaldehyde to produce deposits of paraformaldehyde. The adsorbed or polymerized formaldehyde would have the effect of giving a slow kill on the surface after the concentration in the room had fallen (cf. Kingston, Lidwell & Noble, 1962), but possibly not to a useful extent. That slow evolution probably occurs does however mean that adsorption has stopped, so that the concentration of formaldehyde in the boundary layer will not be below that in the main bulk of the air.

Water vapour is known to be taken up in large amounts by many natural

materials, and the quantity of water required to raise the humidity in a room by a given amount cannot therefore be predicted. Air saturated with water vapour contains, at 20° C., 485 g. of water per 1000 cu.ft., or about 0·85 pints. The quantities suggested (Public Health Laboratory Service, 1958*b*) for vaporization by electrical heating are 1000 ml. of water and 500 ml. of formalin for each 1000 cu.ft., i.e. a total of 1300 ml. of water. This would saturate perfectly dry air 2·7 times. The results of Expts 7 and 8 show that, at least when starting with a relative humidity of 50 %, this amount is adequate. The recipe given for use with permanganate suggests using 500 ml. of neat commercial formalin for 1000 cu.ft., stating that owing to the formation of water from the formaldehyde no further water is needed. Since the specific gravity of commercial formalin is very nearly 1, 200 g. of formaldehyde and 300 g. of water are available. The stoichiometric equation shows that a negligible amount of water would be produced by the oxidation of the formaldehyde. Thus only about 300 g. of water would be available, enough to produce 62 % saturation of perfectly dry air. The measurements show that this much smaller amount is not adequate. The increase in the percentage relative humidity was only of the order of 25. Though the median relative humidity in centrally heated buildings in England is likely to be about 45 %, relative humidities down to 30 % will occur with reasonable frequency (see Kingston & Noble, 1964). An increase of up to 55 would therefore be necessary to raise the relative humidity to the optimum of 80–90 %. When the permanganate method was used with twice the suggested quantities the rise in the relative humidity was still inadequate (Expt 10). Since we found that if appreciable quantities of water were added to the formalin before adding the permanganate, the solution was not all boiled off, we conclude that the permanganate method does not vaporize enough water for optimum fumigation conditions.

Effect on test contamination

In Expt 1 (Table 2) neither the dried broth drops nor the dust samples were sterilized. The specimens exposed on the floor were least affected, survival of the organisms in dust being about 1 %, and in dried broth drops about 10 %. Conditions were unusually dry, and owing to inadequate sealing the formaldehyde concentration fell rapidly.

In the main experiment four cubicles were used (Table 2, Expts 2–5), two being given a 3 hr. exposure to formaldehyde, and two a 24 hr. exposure. The bacteriological results are set out in Table 4. Also in this table are the results of the experiment carried out in a room at the Central Public Health Laboratory (Table 3, Expt 10). In all these experiments the formaldehyde was vaporized by reaction with permanganate in the quantities recommended by the Public Health Laboratory Service (1958*b*), with the exception of Expt 10 in which twice the quantities were used.

The results show that on no occasion was the survival after exposure more than 0·1 %, and that it was generally much less than this. The specimens from which organisms could be recovered were not uniformly distributed, the majority of them occurring in the cubicle of Expt 3 (11 out of 17). Taking all the cubicles

Table 4. *Survival of test contamination when exposed to formaldehyde in rooms*

Period exposed (hr.)	Ref. for Table 2 (Expt no.)	Organism	Nature of specimen	Organisms per prepared specimen (millions)	Survival as percentage of counts at start of experiment					% for 3 cols.†
					Control*	Floor	Window-sill	High	Door	
Isolation cubicles										
3	2	*Staph. aureus*	Dust	100	35	N.G., —‡	N.G., N.G.§	N.G., N.G.	N.G., N.G.	0·0002
			Drop	20	120	0, 0‖	0, 0	0, 0	0, 0	—
		Ps. aeruginosa	Drop	20	18	0, 0	0, 0	0, 0	0, 0	—
3	3	*Staph. aureus*	Dust	100	35	N.G., N.G.	N.G., N.G.	N.G., 0·0001	N.G., N.G.	0·0002
			Drop	20	120	0·095, 0·056	0·069, 0	⩽ 0·002, 0	0, 0	—
		Ps. aeruginosa	Drop	20	18	⩽ 0·002, ⩽ 0·002¶	0·0060, 0·0060	⩽ 0·002, ⩽ 0·002	0, 0	—
24	4	*Staph. aureus*	Dust	100	15	N.G., N.G.	N.G., N.G.	N.G., N.G.	N.G., N.G.	0·0002
			Dust (Bijou)**	50	15	—, —	—, —	N.G., N.G.	—, —	0·0002
			Drop	20	93	⩽ 0·002, 0	0, ⩽ 0·002	0, 0	0, 0	—
		Ps. aeruginosa	Drop	20	18	0, 0	0, 0	0, 0	0, 0	—
24	5	*Staph. aureus*	Dust	100	15	N.G., 0·0002	N.G., N.G.	N.G., N.G.	N.G., N.G.	0·0002
			Dust (Bijou)**	50	15	—, —	N.G., N.G.	—, —	—, —	0·0002
			Drop	20	93	—, 0·0089	0, ⩽ 0·002	—, 0	—, 0	—
		Ps. aeruginosa	Drop	20	18	⩽ 0·002, 0	0, 0	0, 0	0, 0	—
Laboratory room								Bench		
3	10	*Staph. aureus*	Serum dust	800	86	N.G., N.G.	—	N.G., N.G.	—	0·00003
			Broth dust	300	37	N.G., N.G.	—	N.G., N.G.	—	0·0001
			Serum drop	2	82	0, 0, 0	—	0, 0, 0	—	—
			Broth drop	1	130	0, 0, 0	—	0, 0, 0	—	—
		B. subtilis	Spore foil	0·0003	—	0, 0, 0	—	0, 0, 0	—	—
			Spore foil	0·3	—	⩽ 0·09, ⩽ 0·09, ⩽ 0·09	—	0, ⩽ 0·09, ⩽ 0·09	—	—

* The control specimens give the survival found from specimens not exposed to the disinfectant procedure. They differ from the test specimens in the temperature and humidity to which they were exposed, as well as in the absence of formaldehyde.

† See under § below.

‡ Not done.

§ No growth when the specimens were plated out. These dust samples could not be incubated entire, as carry-over of formaldehyde caused inhibition. They might therefore be sterile or have a percentage survival equal to or less than the value given in the column headed '% for 3 cols.', which is that which would have resulted from three colonies being found at the lowest dilution plated.

‖ A zero indicates no growth when the elution fluid and the strip were incubated.

¶ ⩽ 0·002, etc.: survival equal to or less than the stated figure. There was growth of the appropriate organism when the strip and elution fluid were incubated, but no colonies were found from the lowest dilution plated. The stated figure corresponds to 3 colonies at the lowest dilution.

** The dust was in a screw-capped bottle with a small hole pierced in the lid, see text.

The specimens showed a percentage survival in preparation (cf. Table 1) as follows. Isolation cubicles: *Staph. aureus* drop, 100 %; *Ps. aeruginosa* drop, 37 %. Laboratory room (*Staph. aureus*): serum dust, 41 %; broth dust, 34 %; serum drop, 46 %; broth drop, 20 %.

together, 8 of the positive specimens came from the floor, 5 from the window-sill, 4 from the high shelf (all in cubicle 3) and none from the table near the door.

DISCUSSION

The results of our laboratory experiments (Table 1) can usefully be supplemented with the much more extensive series of the Committee on Formaldehyde Disinfection (Public Health Laboratory Service, 1958*a*). Since the significance of their results has sometimes been misunderstood, those which bear on the problem of terminal disinfection are summarized in Table 5. The test objects were cotton threads on to which about 10^4 organisms, either in 1 % gelatin or 90 % horse serum, had been dried. When these were exposed, hanging free in an atmosphere containing formaldehyde at a temperature of 20° C., times for rendering 45 % of the threads sterile were found, and are given in the table. It was also found that

Table 5. *Time for sterilization of* 45 % *of impregnated cotton threads*

(Public Health Laboratory Service, 1958*a*.)

Organism	Conditions	Vehicle	Time (min.)
Micrococcus	58 % R.H., 1 mg./l.*	1 % gelatin	29
Micrococcus	58 % R.H., 1 mg./l.*	90 % horse serum	276
Micrococcus	Vapour of 40 % formalin (1·75 mg./l.*)	90 % horse serum	54
Myco. tuberculosis (avian)		90 % horse serum	60
B. subtilis (spores)		90 % horse serum	173

Each thread had about 10^4 organisms dried on to it in the vehicle shown.
R.H.: relative humidity.
* Weight of formaldehyde per litre of air.

increasing the relative humidity from 58 % to 80–90 % approximately halved the time, and that exposing threads under three layers of blanket approximately doubled it. It was also found that cotton threads on which a 1 % suspension of variola major crusts in 90 % monkey serum had been dried were sterilized when exposed for 24 hr. in a disinfection cabinet with an initial concentration of about 3 mg. formaldehyde per litre, but that whole scabs were not. These results show that heavily contaminated test objects, even when moderately protected, were fairly readily sterilized by formaldehyde vapour, and that this disinfectant action was non-specific. A large mass of organic matter (whole smallpox scabs, dried horse serum) slowed the process down, presumably by delaying penetration. A high humidity potentiated the action of the formaldehyde, which was sometimes inactive at 32 % humidity.

The results of the formaldehyde and water-vapour concentrations found in the cubicles and laboratory room (Table 2) show that where the room was properly sealed the formaldehyde concentration was satisfactory up to 3 hr. after vaporization. The permanganate method did not vaporize enough water to achieve the optimum humidity, but even so a very substantial reduction in contamination was found (Table 4). Organisms in dust were more readily killed than those in dried drops. Specimens showing growth were not uniformly distributed.

Any evaluation of a disinfectant procedure is dependent on the tests simulating

sufficiently closely the conditions under which the disinfectant has to work in practice. If our tests were in fact good models, then our conclusions are that formaldehyde fumigation can be a satisfactory means of decontaminating an isolation cubicle, but that a number of points must be carefully attended to. The cubicle must be thoroughly sealed. In large buildings, considerable pressure differences may be set up by thermal convection and by wind, with the result that the vapour can be very readily sucked up ducting and through cracks under doors, etc. This may produce serious falls in concentration and make other parts of the building uninhabitable. Absolute sterility may never be reliably achieved, and really massive contamination will not be adequately disinfected and must be removed mechanically. Thus cleaning is desirable before fumigation. Bedding, particularly if opened out to the action of the vapour, would probably be effectively disinfected, but it would be preferable to remove it and deal with it separately.

We think that the permanganate method of volatilizing the formaldehyde usually will not vaporize enough water. If there is no alternative means of vaporization, twice the quantities suggested by the Public Health Laboratory Service (1958*b*) should be used, namely for each 1000 cu.ft. two lots of 500 ml. of formalin each reacted with 170 g. of $KMnO_4$. We think that it is preferable to boil off 500 ml. of formalin in 1000 ml. of water by some suitable heating system, or possibly to vaporize it by an atomizing spray. Unless the apparatus can safely be run dry, an electrical cut-out device which is unlikely to fail is necessary, since fogging may make it impossible to control the vaporization by eye. We think that it is probably an advantage to run a fan in a room being fumigated.

It would be convenient if there were some simple means of checking that the fumigation procedure had been satisfactory. Estimation of the formaldehyde and water vapour concentrations at the start of the fumigation and after 3 hr. would probably be the best method. If during this time the formaldehyde concentration did not fall much below 1 mg./l. of air, and if the humidity started at 80–90 % and did not fall below about 60 %, the procedure is likely to have been satisfactory. The technique we used for formaldehyde estimation seemed to us to be satisfactory; another has been suggested by Harry (1959). Bacteriological test pieces are tedious to prepare, difficult to standardize, and often erratic in their behaviour. We therefore included the spore test piece of Beeby & Whitehouse (1965) in some of our experiments, since this avoids many of these disadvantages. Unfortunately, it seemed possible that the action of formaldehyde on this test piece might, in comparison with the experimental contamination, be less affected by the humidity. Since we think the spore test piece to be a less good representation of naturally occurring contamination, we cannot recommend its use here without further investigation. The spore test pieces are made from a fairly resistant form exposed freely to the vapour, whereas natural contamination will be protected by dried fluids, skin scales and so on, through which the formaldehyde must diffuse before it can reach the rather more susceptible vegetative forms.

Lack of knowledge of the relative importance of the many different routes by which diseases can spread makes it impossible to lay down definite rules for deciding when terminal disinfection should be carried out. However, there are

some general principles to be considered. A source of specific recommendations is the report of the American Public Health Association (1965).

Disinfection is no substitute for cleaning. As has been pointed out, substantial pieces of contamination cannot be adequately dealt with by fumigation and must be removed mechanically. Since for other reasons also failures may occur, the more contamination that is removed by cleaning the better. Even the most thorough cleaning, however, will not remove all contamination, though it may be thought that the organisms remaining after a really thorough cleaning will be too inaccessible or too adherent to be likely to reach another susceptible host.

It is often pointless to try to achieve complete sterility. The ordinary hospital environment, however clean initially, is rapidly recontaminated with organisms. In particular, *Staph. aureus* and *Cl. welchii* are carried by about half the adult population and are therefore continually being disseminated. There is seldom any point in ensuring a total, but temporary, absence of these organisms. Thus the only occasions on which fumigation could be of value are if dangerous organisms of high infectivity are present, and if these organisms are not carried by a high proportion of normal people in hospital. It could be argued that strains of *Staph. aureus* which were thought to be unusually dangerous should be put into this category. Apart from the difficulty in deciding which strains are usually dangerous, there is some evidence that *Staph. aureus* is not readily acquired from fomites where it is present in small numbers (Gonzaga, Mortimer, Wolinsky & Rammelkamp, 1964). The only disease for which a general recommendation for fumigation has been made is smallpox (Ministry of Health and Scottish Home and Health Department, 1964). Where specially dangerous organisms are concerned, it may be advisable to fumigate before cleaning, as well as after, to give the greatest possible protection to the cleaners.

There are a number of special cases to be considered. Certain peculiarly susceptible patients have to be cared for in an environment which is as free as possible from bacterial contamination. Since extraordinary precautions are taken to reduce recontamination, there may be a case for ensuring that the room is as sterile as possible initially. Organisms capable of multiplying in the environment must be considered separately, since if any were left they might increase to serious levels again, whereas ordinary contamination is continually reduced by the natural death-rate of the organisms. Bacteria do not multiply in the dry state, and only those which are capable of multiplying in the more or less enriched tap-water of sinks and similar places can proliferate in the environment. The most important example is *Ps. aeruginosa*, but other organisms such as *Flavobacterium meningosepticum* can cause trouble in this way. However, since they will only be multiplying in localized areas, special measures directed against the damp places may be more appropriate.

This discussion has centred on the possible use of formaldehyde fumigation in the type of room in which the tests were carried out, namely isolation cubicles. Other buildings may differ in important ways, and it would be necessary to show for them whether or not conditions were satisfactory. The surface/volume ratio and the absorbancy of the walls and contents would affect the amount of formalde-

hyde and water needed to bring the air concentrations up to reasonable levels; this, however, would be checked by estimating the formaldehyde concentration and relative humidity. More seriously, a large mass of absorbent material might keep the formaldehyde and water vapour concentrations in the boundary layer over it, and hence over the bacteria, at a low level. The buildings must be capable of being effectively sealed. Temperature may be of importance, though the Public Health Laboratory Service (1958*a*) found little effect under their conditions. However, the equilibrium vapour pressure of formaldehyde over paraformaldehyde becomes low below 10° C. and this may impose a limitation, and there is the possibility of serious loss by condensation on cold surfaces even though the air temperature might be adequate. Attention must also be paid to the nature of the contamination, since formaldehyde will not readily penetrate large masses of organic material. For several of these points see Lancaster, Gordon & Harry (1954), Harry (1961) and Harry & Hemsley (1964).

SUMMARY

The conditions for satisfactory fumigation with formaldehyde were investigated using suspensions of *Staph. aureus* and *Ps. aeruginosa* dried as drops on strips of polythene or after mixing with cotton dust. In laboratory experiments with controlled concentrations of formaldehyde and water vapour, satisfactory results were obtained at 86 % humidity, but at 32 % humidity the formaldehyde was virtually inactive.

Measurements under field conditions showed that the permanganate method of vaporizing formaldehyde did not vaporize enough water to produce optimum conditions for sterilization, and that great care was necessary in the sealing of rooms. When sterilization was incomplete the surviving organisms were not uniformly distributed within the room.

In the limited circumstances in which fumigation with formaldehyde is thought to be essential, the process should be controlled by estimating the formaldehyde and water vapour concentrations initially and after 3 hr.

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