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Corrigendum

DERRICK BAXBY and C. J. M. RONDLE, *J. Hyg., Camb.* (1968) **66**, 191–205.

On page 204, in reference to Easterbrook, K. B. (1962), for *Virology* **27**, 245, *read Virology* **17**, 245. In reference to Easterbrook, K. B. & Davern, C. I. (1963), for *Virology* **17**, 509 *read Virology* **19**, 509.

J. Hyg., Camb. (1968), **66**, 319
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A modification of the growth-inhibition test and its use for detecting *Mycoplasma mycoides* var. *mycoides*

BY G. DAVIES* AND W. C. S. READ*
East African Veterinary Research Organization, Muguga, Kenya

(*Received* 27 *December* 1967)

INTRODUCTION

The growth-inhibition test is, by now, a well recognized method of identifying mycoplasma species. It has not, however, been widely used for the routine identification of *Mycoplasma mycoides* var. *mycoides*, the causal agent of contagious bovine pleuropneumonia (CBPP). This paper describes a modification of the test designed to facilitate the screening of large numbers of mycoplasma ioslates for *M. mycoides* var. *mycoides* and experiments to check the specificity of the test when used for this species.

MATERIALS AND METHODS

Liquid media

Tryptose serum broth (Newing & Macleod, 1958) as modified by Gourlay (1964) was used. Preparation of this broth has been fully described by Brown, Gourlay & Macleod (1965). The broth included penicillin (100 i.u./ml.) and thallium acetate (1 part in 2000) as bacteriostatic agents. The dextrose component was omitted from some batches of broth as *M. mycoides* var. *mycoides* maintains its viability for a longer period in such a medium.

Solid medium

Tryptose serum agar (Gourlay, 1964) was used. This was prepared in two parts:

A. Tryptose agar

Bacto tryptose (Difco)	2·00 % (w/v)
Sodium chloride	0·50 % (w/v)
Glycerol	0·50 % (v/v)
Anhydrous di-sodium phosphate (Na_2HPO_4)	0·25 % (w/v)
Distilled water to	100·00 %

B. Pig serum and additives

Inactivated pig serum	30 ml.
Dextrose	5 ml. (10 %, w/v)
Bacto yeast extract (Difco)	1 ml. (10 %, w/v)
Crystalline penicillin G (Glaxo)	100,000 units
Thallium acetate	1 ml. (1 % solution)

Part A was prepared and kept in stock until needed.

Part B was prepared immediately before use and filtered through Seitz E.K.

* Seconded to the East African Veterinary Research Organization from the Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Weybridge, Gt Britain.

pads before being added to the melted part A in the ratio of 100 vols. part A to 36 vols. part B. Care was taken to use fresh pig serum and to avoid heating it for more than 30 min. at 56° C. as *M. mycoides* var. *mycoides* has been found to be extremely sensitive to changes in the serum component of solid media. In some batches of solid medium the penicillin and thallium acetate were omitted.

Antisera

These were prepared in rabbits using the method of Lemcke (1964). All sera were heated to 56° C. for 30 min. before use.

Precipitin tests

These were carried out as described by Turner (1962) using supernatant from centrifuged broth cultures as antigen and rabbit antisera as antibody.

The growth-inhibition test

The growth-inhibition test used for identification was modified to deal with large numbers of strains.

Tryptose serum agar plates were dried and the back of the plates marked off into squares $\frac{1}{2} \times \frac{1}{2}$ in. The cultures to be tested were inoculated into tryptose broth devoid of dextrose; incubated cultures showing a fine opalescence were used for the test. One loopful of each culture was spread over the surface of the medium so as to cover the area of a marked square. The squares were labelled with the identity of the cultures and the plates allowed to dry at room temperature for 15–30 min. A loopful of *M. mycoides* var. *mycoides* antiserum was placed on the medium in the centre of each square using a 2–3 mm. diameter platinum loop bent at right angles to the stem, and allowed to dry on the plates at room temperature before incubation at 37 °C. in a moist atmosphere. Plates were examined after 48–72 hr. incubation and cultures that showed a central zone of inhibition of growth (see Plate 1) were recorded as *Mycoplasma mycoides* var. *mycoides*.

RESULTS

The specificity of the growth-inhibition test for identification of Mycoplasma mycoides *var.* mycoides

Differentiation of Mycoplasma mycoides *var.* mycoides *from other* Mycoplasma *species*

Rabbit antisera, produced against the Gladysdale strain of *M. mycoides* var. *mycoides*, and normal rabbit serum were tested against cultures of the following organisms, using a growth-inhibition test: *M. mycoides* var. *mycoides* (Gladysdale strain), *M. mycoides* var. *capri* (Longley strain (Longley, 1951)), *M. laidlawii* A. (PG 8), *M. bovirhinis* (PG 47), *M. agalactiae* var. *bovis* (*M. bovimastitidis*) (Donetta; PG 45), unnamed mycoplasma (Squire; PG 49), unnamed mycoplasma (N 29; PG 50), unnamed mycoplasma (D 12; PG 51).

The normal rabbit serum failed to produce a zone of inhibition of growth in any of these cultures. *M. mycoides* var. *mycoides* antiserum produced a clear zone of inhibition in the culture of *M. mycoides* var. *mycoides*, but no zone of inhibition with any of the other cultures.

The response of various strains of M. mycoides *var.* mycoides *to the growth-inhibition test*

Four strains were examined:

(1) T_1 (46th passage): isolated in Kajiado, Kenya, and used as a vaccine strain in east Africa. (Piercy & Knight, 1957) (Brown *et al.* 1965).

(2) KH_3J (85th passage): isolated in the Sudan and used as a vaccine strain there and in West Africa.

(3) Archers Post (2nd passage): isolated in Northern Kenya (Bygrave, Moulton & Shifrine, 1968).

(4) Gladysdale: isolated in Australia and since then passaged continuously in cattle as infected lung material.

Rabbit antiserum to the Gladysdale strain of *M. mycoides* var. *mycoides* was used on the above strains in the growth-inhibition test. In each case a clear zone of inhibition of growth was caused by this serum. No colonies grew within the zone and normal rabbit serum produced no effect. This suggests that *M. mycoides* var. *mycoides* can be identified by this test irrespective of the strain or the number of subcultures it has undergone.

The identification of Mycoplasma mycoides *var.* mycoides *in a mixed culture*

Twenty-four-hour broth cultures of *M. mycoides* var. *mycoides* and *M. bovirhinis* were mixed in the following ratios: 3:1, 1:1 and 1:3. The mixed cultures were plated and tested with serum prepared against *M. mycoides* var. *mycoides* (Gladysdale strain). In each case a distinct zone of inhibition of growth was visible even though there was a fine growth (presumably of *M. bovirhinis*) within the zone.

The effect of the concentration of organisms on the clarity or size of the zone

Tenfold dilutions of a 24 hr. culture of *M. mycoides* var. *mycoides* were made in tryptose broth. Each of these dilutions was plated and tested for inhibition of growth by *M. mycoides* var. *mycoides* antiserum as before. Each dilution of culture which produced growth on the plates (up to 10^{-5} dilution) showed a zone of inhibition of growth. The zones did not vary in size and in each case there was no visible growth within the zone. This suggests that, using this modification of the test, the concentration of organisms is not critical.

Comparison of the results obtained with the growth-inhibition test and the precipitin test

A total of 110 mycoplasma strains isolated from animals involved in vaccine trials and animals slaughtered during a field outbreak of CBPP were examined using the above two tests. The results recorded in Table 1 show complete correlation between the two tests. In addition, the precipitin test was carried out, using anti-*M. mycoides* serum, on the seven bovine mycoplasma serotypes tested above *M. mycoides* var. *mycoides* was the only one to give a positive reaction.

Table 1. *Comparison of two tests to distinguish* Mycoplasma mycoides *var.* mycoides

		Growth-inhibition	Precipitin
Experiment A (14 animals infected with Gladysdale strain *M. mycoides*)	+ve	68	68
	−ve	15	15
Experiment B (8 animals infected with T_1 strain *M. mycoides*)	+ve	14	14
	−ve	4	4
Field outbreak in Northern Kenya	+ve	9	9
	−ve	0	0

(The figures indicate the number of strains tested.)

DISCUSSION

The growth-inhibition test for identifying mycoplasma species was developed by Edward & Fitzgerald (1954) from an observation by Priestley (1952). It appears to be more specific than the complement fixation and indirect haemagglutination tests (Clyde, 1964) and is consistent with nucleic acid homology (Reich *et al.* 1966). Various techniques have been tried but the filter-paper-disk method developed by Huijmans-Evers & Ruys (1956) and used in human mycoplasma identification by Clyde (1964) is the one universally used. The disadvantages of this technique are the large amounts of serum used and the liability of the sera to bacterial contamination. In addition, the size of the zone of inhibition is influenced by the density of the colonies on the plate (Clyde, 1964). Stanbridge & Hayflick (1967) have attempted to overcome the first two disadvantages by freeze-drying paper disks previously saturated with antisera.

In this laboratory we have been screening very large numbers of mycoplasma strains for *M. mycoides* var. *mycoides* from cultures made from the carcasses of cattle used in vaccine trials for CBPP. It became apparent that there was an extensive mycoplasma flora in these cattle and the modification of the growth-inhibition test described in this paper was developed to identify these strains. Our experience has been that it reduces the number of manipulations to a minimum and thus reduces the risk of bacterial contamination of the sera. It is also economical with serum. The zones of inhibition produced are very distinct (see Plate 1) and as they do not depend to any great extent on diffusion of the serum their size remains constant irrespective of the density of growth on the plate.

The growth-inhibition test has been used for the identification of *M. mycoides* var. *mycoides* by workers at Farcha (unpublished work, reported at the FAO meeting, Khartoum, 1967). It has also been used by Leach (1967) to differentiate the various mycoplasmas isolated from cattle. The present work confirms Leach's findings that the test distinguished between *M. mycoides* var. *mycoides* and the other known bovine mycoplasmas. (The only bovine mycoplasma not examined was *M. bovigenitalum*, which was unavailable.)

There is no published work on the results of the growth-inhibition test when used with various strains of *M. mycoides* var. *mycoides*. The present work suggests that strains react identically to the test regardless of whether they are recently isolated or attenuated strains.

The precipitin test has been used by workers in Australia (Turner, 1962), particularly to identify *M. mycoides* var. *mycoides* antigens in lung lesions where cultural examination was impossible. The present results show complete agreement between the two tests.

We have used the growth-inhibition test for epidemiological studies in CBPP and it has distinguished mycoplasma other than *M. mycoides* var. *mycoides* in cultures from the respiratory tract of cattle which had typical CBPP lesions and from which *M. mycoides* var. *mycoides* was isolated. Mycoplasmas other than *M. mycoides* var. *mycoides* can also be isolated from the macroscopically normal lungs of experimental cattle in East Africa and these findings suggest that it is essential to use an identification test such as the growth-inhibition test on all mycoplasma strains from such cattle. Until now it has been customary to rely on the characteristic 'thread' growth of *M. mycoides* var. *mycoides* in fluid media to identify the organisms, but Turner (1959) has pointed out that the thread phase is only seen in cultures derived from small inocula and Razin, Cosenza & Tourtellotte (1967) have demonstrated thread phases in a number of other mycoplasmas.

SUMMARY

A modification of the growth-inhibition test for identifying *Mycoplasma* species is described. The modification simplifies the screening of a large number of strains for one species. Experiments showed that it was effective and specific when used to identify *M. mycoides* var. *mycoides*. Its use in studying the epidemiology of contagious bovine pleuropneumonia is discussed.

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We wish to thank Dr R. H. Leach for the various bovine mycoplasma cultures.

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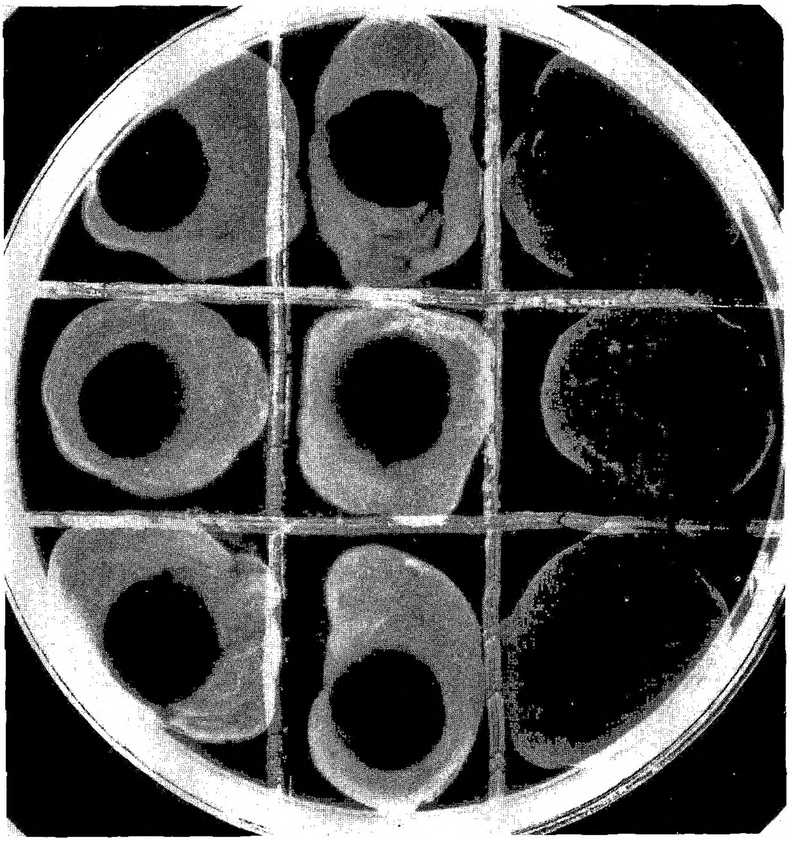
EXPLANATION OF PLATE

Mycoplasma strains isolated from the respiratory tract of a cow. The six cultures on the left show zones of inhibition of growth which identifiy them as *M. mycoides* var *mycoides*. (Stained neutral red.)



G. DAVIES AND W. C. S. READ

(*Facing p.* 324)

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Bacteria in a hospital nursery: laboratory and clinical studies

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(*Received* 30 *December* 1967)

INTRODUCTION

Of the many investigations for the presence of bacteria and of clinical sepsis in hospital nurseries most have focused attention on the staphylococcus. The publications of Rountree & Barbour (1950); Edmunds, Elias-Jones, Forfar & Balf (1955); Hutchison & Bowman (1957); Gillespie, Simpson & Tozer (1958); Plueckhahn & Banks (1958); Williams (1961), and many others, are testimonies to the interest engendered by this organism. In contrast, published accounts of the ecology of the Gram-negative intestinal bacilli have been more infrequent. Laursen (1963) and Kresky (1964) described the colonization of neonates with Gram-negative bacilli, as also have Shallard & Williams (1965, 1966); and Sarkany & Gaylarde (1967) included these organisms in their account of the skin flora of the immediate newborn. Studies illustrating the epidemiological properties of the Gram-negative intestinal bacilli are being more frequently reported, the most comprehensive being that of Laursen (1962) and Reinarz, Pierce, Mays & Sandford (1965). The increasing importance of the Gram-negative bacilli in hospital nurseries has been summarized. (Editorial. *Am. J. Dis. Child.* 1961.)

The present study took place from August to October 1964, and was a preliminary to a subsequent trial on the effects of antiseptics in two hospital nursery units, of which this unit (Western General Hospital) is one. It was required to know (*a*) the rate of colonization of infants with *Staphylococcus pyogenes* and Gram-negative bacilli under non-epidemic conditions, (*b*) the staff carriage of these bacteria, (*c*) their incidence in the nursery environment, and (*d*) the state of clinical infection due to these organisms. This information was required to establish normal patterns in the ecology of *S. pyogenes* and Gram-negative bacilli in a hospital nursery where no antimicrobial agent was used for infants other than surgical spirit treatment of the umbilical stump and plain soap for washing purposes, plain soap only being used for staff handwashing. This paper also discusses the present position of *S. pyogenes* in a nursery with particular attention to the changes in ecology resulting from the introduction of the penicillinase-resistant penicillins.

MATERIALS AND METHODS

Laboratory investigations

Examination of infants, attending staff and ward environment

Infants. Swabs were taken from the nose, post-nasal space and umbilicus of each infant, up to three times during the first 7 days of life. Swabbing of long-stay infants was continued beyond the seventh day. For nasal and per-nasal sampling small diameter cotton-wool swabs were used. For sampling the umbilicus the usual adult-type throat swab was employed.

Attendant staff. Broth-moistened swabs of the nose, together with hand-impression plates were taken on repeated occasions. The hand impressions were obtained by placing the tips of the digits and the palms of both hands on the surface of solid culture media.

Ward environment. Repeated sampling of ward fixtures and fittings, walls, floors, etc., was done using Sellotape transfers (Thomas, 1961) and broth-moistened swabs. The bacterial content of the air was measured using the 'Casella' slit sampler, sieve sampler (duBuy & Crisp, 1944) and settle plates. Five cu. ft. volumes of air were sampled on each occasion using the slit sampler, and 12·5 cu. ft. volumes with the sieve sampler. Settle plates were placed on the floor for one hour in different parts of the nursery. These examinations coincided with the sampling of infants and staff.

Media

All swabs were inoculated on 5% horse blood agar and MacConkey agar which were incubated for 18 hr. at 37° C. and examined for growth. Each swab was inoculated in a zig-zag manner covering a half plate. The same media were also used for the hand impressions and Sellotape transfers. For air sampling horse blood agar only was used.

Identification of bacterial growth

Staphylococcus pyogenes. A single representative colony from each blood agar plate was subjected to the slide test for coagulase production (Cadness-Graves, Williams, Harper & Miles, 1943) and to the tube method (Cowan, 1938).

Gram-negative bacilli. Identification was by the macroscopic appearance of colonial growth on culture supported by the Gram stain. No attempt was made in this preliminary study to measure the incidence of the different genera, except where clinical infection supervened.

Phage typing of Staphylococcus pyogenes

This was done by the method of Blair & Williams (1961) using phage filtrates at a concentration 100 times the routine test dilution (100 RTD). Where no result was obtained at this dilution undiluted phage filtrate was used and only when this failed to yield lysis was the strain recorded as 'non-typable'. The classification of *S. pyogenes* phage typing patterns into phage groups followed that proposed by Blair & Williams (Table 1).

Sensitivity to antibiotics of Staphylococcus pyogenes

Strains were tested for sensitivity to penicillin (1 unit in disk), streptomycin (10 μg), chloramphenicol (25 μg.), tetracyline (25 μg.), erythromycin (10 μg.) and methicillin (10 μg.) by the filter paper disk method (Gould & Bowie, 1952). The organism was recorded as sensitive when the diameter of the zone of inhibition of growth was 15 mm. or greater, and as resistant when 14 mm. or less, the diameter of the disk being 6·25 mm.

Table 1. *Classification of* Staphylococcus pyogenes *into phage groups* (*Blair & Williams*, 1961)

Phage group	Lysed by bacteriophages
Group I	29, 52, 52 A, 79, 80
Group II	3 A, 3 B, 3 C, 55, 71
Group III	6, 7, 42 E, 47, 53, 54, 75, 77, 83 A
Group IV	42 D
Miscellaneous	81, 187
Mixed	Lysis by phages from more than one group

Note: where phage pattern was 80/81 the strain was placed in group I.

For convenience in handling the different antibiotic-sensitivity patterns encountered, a system of coding was adopted:

Category A. Strains sensitive to chloramphenicol, erythromycin, and methicillin, and resistant to penicillin, streptomycin and tetracycline.

Category B. Strains resistant to penicillin only.

Category C. Strains sensitive to all the antibiotics tested, together with the occasional strain possessing an unusual sensitivity pattern. These latter were mostly encountered in infants, from whom very few multiple-sensitive strains were isolated, and showed resistance to chloramphenicol or to erythromycin, or to tetracycline only.

Interpretation of cultures

The presence of small numbers of pathogenic bacteria recovered from the nose, and from the skin and fomites, can not necessarily be considered of epidemiological significance (Wallace & Duguid, 1952; White, 1961; Gonzaga, Mortimer, Wolinsky & Rammelkamp, 1964). For this reason a system of scoring the numbers of colonies yielded from the samples was employed in this survey. Growth on blood agar and MacConkey agar was scored as follows:

Swabs from infants and attendant staff. More than 100 colonies of *S. pyogenes* or Gram-negative bacilli constituted a heavy growth and was scored + + +, 30 to 99 colonies a moderate growth and scored + +, and 10 to 29 colonies a scanty growth, scored +. Less than 10 colonies, and a + growth in the presence of other bacteria was not considered of epidemiological significance and has not been included in the results.

Hand impressions, Sellotape transfers and swabs of ward surfaces. More than eight colonies of either *S. pyogenes* or Gram-negative bacilli constituted a heavy growth and was scored + + +, 5 to 8 colonies a moderate growth scored + +, and 2 to 4 colonies a scanty growth scored +. Less than 2 colonies was not considered significant and has not been included in the results. A + growth was considered significant even when other organisms were present.

Slit and sieve sampler, and settle plates. The total bacterial count was recorded, and all *S. pyogenes* isolated were considered of epidemiological significance.

Clinical investigation

Independently of the laboratory investigations, bacteriological examinations were arranged on any infant showing clinical evidence suggesting infection. A careful record was kept of suspected infections and any treatment given.

Description of nursery

Two main receiving wards each of up to twenty cots, together with a premature baby unit of three to six cots or incubators, a wash room, isolation ward, and a corridor separating the two receiving wards constituted the nursery unit. These areas were all examined bacteriologically.

Routine infant care and ward hygiene

Infants were bathed with soap and water at delivery and every fourth day thereafter. Sponging with water only was done each day. The umbilical stump was treated with spirit after each napkin change and was left without any occlusive dressing. Ward floors were vacuum-cleaned daily and washed weekly with 'Savlon'. Furniture, cots, window ledges, etc., were 'damp-dusted' with 'Savlon' daily. No special treatment was employed for cot sheets and blankets; baths were cleaned with 'Savlon' after each bathing.

RESULTS

Incidence of bacteria

Staphylococcus pyogenes

Infants. Two hundred and twenty-two infants were examined, from whom a total of 1753 swabs were taken. There was a progressive increase in the isolation rate of staphylococci from the three sites sampled (Fig. 1), reaching a peak on the sixth day in normal infants (i.e. those which were discharged on the seventh day of life). The umbilicus was the most frequently colonized, 73·1 % of swabs examined on the sixth day yielding these organisms.

The low rate of isolation from the nose during the first 3 days of life is in marked contrast to the results of other workers (Cunliffe, 1949; Gillespie *et al.* 1958) who obtained 44–55 % carriage rate even on the second day. Our finding that 73·1 % of umbilical swabs yielded staphylococci on the sixth day is significantly greater than that of Edmunds *et al.* (1955), where only 40·1 % of umbilical samples were positive for this organism on the eighth day. However, Hutchison & Bowman (1957) obtained a 77·2 % prevalence on the third day. That the umbilicus was colonized before the nose agrees with the findings of Gillespie *et al.* (1958) and Hurst (1960*a*).

The slow acquisition of nasal carriage observed by us may be attributable to the use of penicillinase-resistant penicillins, introduced in 1960. Traces of these penicillins in the ward air may be inspired, thus delaying nasal colonization. That the umbilical carriage rates were unaffected may be due to the smaller quantity of air impinging on the umbilicus, and hence much less exposure to antibiotic.

Attendant staff. Nine of 51 subjects (18%) were nasal carriers, and 27 (53%) yielded staphylococci from their hands (Table 2). Nasal carriage is lower than has

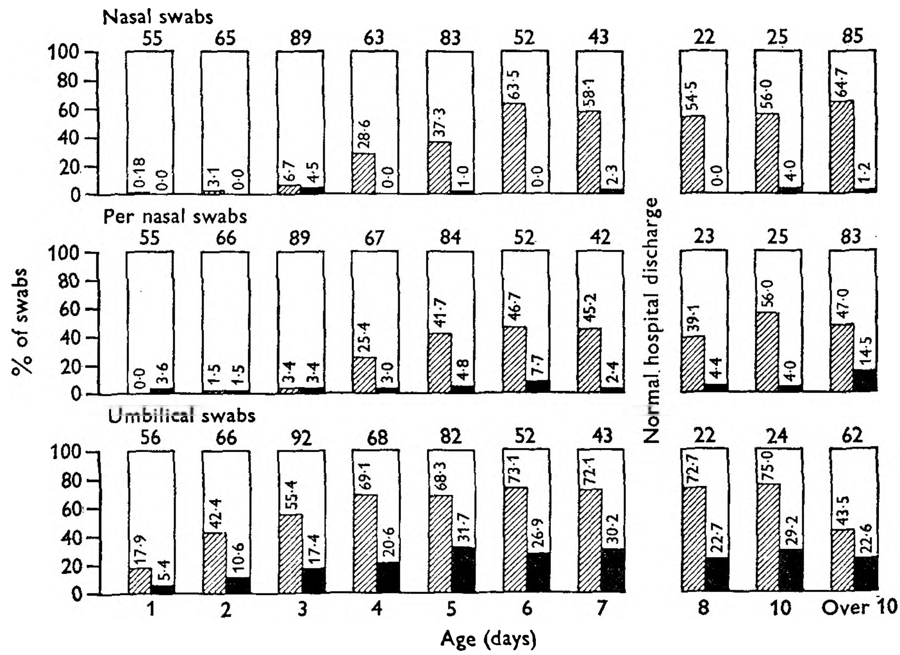

Fig. 1. Infants. Percentage isolations of *Staphylococcus pyogenes* and Gram-negative bacilli. Figures at head of columns denote the number of swabs taken. ▨ *Staphylococcus pyogenes*; ■ Gram-negative bacilli.

Table 2. *Carriage by staff of* Staphylococcus pyogenes *and Gram-negative bacilli*

	Total number	Number positive for *Staph. pyogenes*	Number positive for Gram-negative bacilli
Staff investigated	51	28 (55)*	8 (16)
Nasal swabs taken	116	18 (16)	0
Hand swabs taken	116	35 (30)	10 (9)
Nasal carriers		9 (18)	0
Hand carriers		27 (53)	8 (16)

* Figures in parentheses indicate percentages.

been previously recorded (Ludlam, 1953; Edmunds *et al.* 1955; Hutchison & Bowman, 1957; Monro & Markham, 1958; Poole, 1960), where the findings ranged from 21 to 58%. This low nasal carriage may be due to exposure to the new penicillins as postulated in the case of infants. Reports of the incidence of hand carriage are infrequent, though Love *et al.* (1963) found 11·8% of nurses' hands yielded staphylococci after handling a colonized infant, and Plueckhahn & Banks (1958) found 28 out of 210 nurses' hands (13·4%) positive for staphylococci.

Table 3. *Ward environment examinations. Bacterial contamination of ward fixtures and fittings, and bacterial content of air*

	Number of samples	Number positive for *Staph. pyogenes*	Number positive for Gram-negative bacilli	Total counts Max.	Total counts Min.	Total counts Aver.
Sellotape transfers	131	41 (31)*	7 (5)			
Broth-moistened swabs	103	6 (6)	4 (4)			
Air						
Slit sampler	30	16 (53)	NR	320†	6†	77†
Sieve sampler	77	51 (66)	NR	320‡	11‡	112‡
Settle plates	67	27 (40)	NR	176	3	61

* Figures in parentheses indicate percentages.
† Counts per 5 ft.^{3}.
‡ Counts per 12·5 ft.^{3}.
NR = not recorded.

Ward environment. Table 3 shows the results of sampling ward surfaces and ward air. Sellotape was more effective than swabs in surface sampling, yielding a 31% isolation rate. The three air examination methods (slit and sieve samplers, settle plates) yielded mean isolation rates as follows:

0·11 *S. pyogenes*-carrying particles per cu. ft. (slit),
0·05 *S. pyogenes*-carrying particles per cu. ft. (sieve),
0·40 *S. pyogenes*-carrying particles per one hour (settle).

Staphylococci constituted 0·7% of the total bacterial count (slit), 0·6% (sieve) and 0·7% (settle). These results are in close agreement with those reported in an earlier study of staphylococcal air contamination in a hospital nursery (Wallace & Duguid, 1952).

Gram-negative bacilli

Infants. Colonization was much less evident than with staphylococci (Fig. 1). The highest incidence of carriage of Gram-negative bacilli was observed in the umbilicus on the fifth day (31·7%). Laursen (1963) found Gram-negative bacilli in 15·6% of umbilical swabs taken after the separation of the cord, which presumably was about the eighth day of life. As in our study, Laursen did not use any occlusive dressings, but she did apply a topical antibiotic powder to the umbilicus in all cases. Shallard & Williams (1966) found 58 % of umbilical swabs taken on the first day of life to contain Gram-negative bacilli, a much more rapid acquisition than observed in this series. The same authors obtained a 36 % carriage rate from the noses of infants less than 24 hr. old, which compares markedly with our inability to recover Gram-negative bacilli from the infants' noses during the first 48 hr. of life. The high prevalence of Gram-negative bacilli reported by Shallard & Williams was possibly due to their daily use of chlorhexidine in infant care, this antiseptic being especially active against Gram-positive flora. They did not state whether the umbilicus was covered or not.

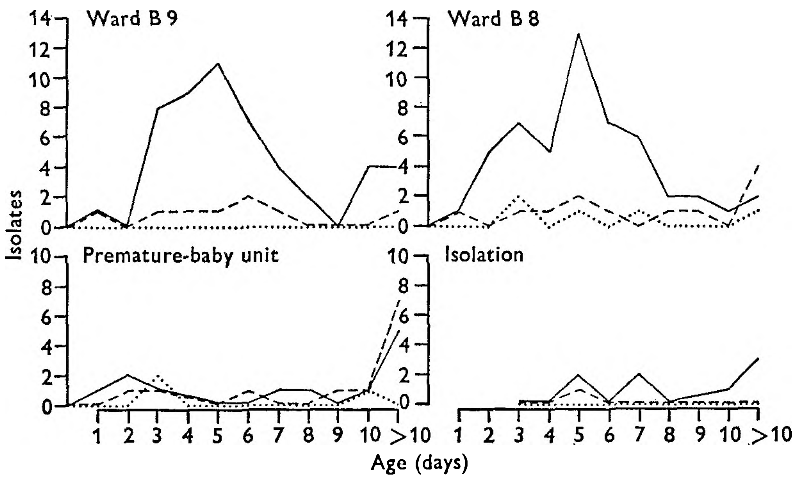

Fig. 2. Gram-negative strains from component wards of nursery (infants).
. . . Nose; ---- post-nasal area; —— umbilicus.

The component units of the nursery were studied, and an increased frequency of isolation of Gram-negative bacilli from those infants in the two main receiving wards was noted (Fig. 2). The frequency of isolation in the two wards (premature baby unit and isolation ward) where special nursing precautions were enforced was negligible.

Attendant staff. No Gram-negative bacilli were isolated from the noses of the staff, but 16 % showed transient hand carriage (Table 2). This is in close agreement with Shallard & Williams (1965), who obtained these organisms from 10 % of hands of staff, and who stated that the noses of the staff 'seldom contained Gram-negative bacilli'.

Ward environment. Only 5 % of surface samples yielded Gram-negative bacilli (Table 3), which contrasts with the 35 % yield-rate from ward surfaces reported by Shallard & Williams (1965). The reason for this marked difference is not apparent. Certainly no antibiotics were used in treating infections caused by Gram-negative bacilli during our study, and this may well have been a factor in the low surface contamination observed.

The incidence of these organisms in the ward air was not measured in this particular investigation. Unpublished observations in the same nursery (Winton, 1965) have shown that the mean isolation rates were:

0·005 Gram-negative bacilli carrying particles per cu. ft. (sieve),

0·20 Gram-negative bacilli carrying particles per one hour (settle).

Gram-negative bacilli constituted 0·09 % of the total air-borne flora (sieve) and 0·15 % (settle). It may thus be stated that Gram-negative bacilli at the time of this study were present in the air in about one-half to one-tenth the concentration of *S. pyogenes*.

The ratio of recovery of *S. pyogenes* to Gram-negative species from surfaces was 4·3 to 1. This is similar to the proportions of these bacterial species in the air, which was 4·7 to 1 (settle plates).

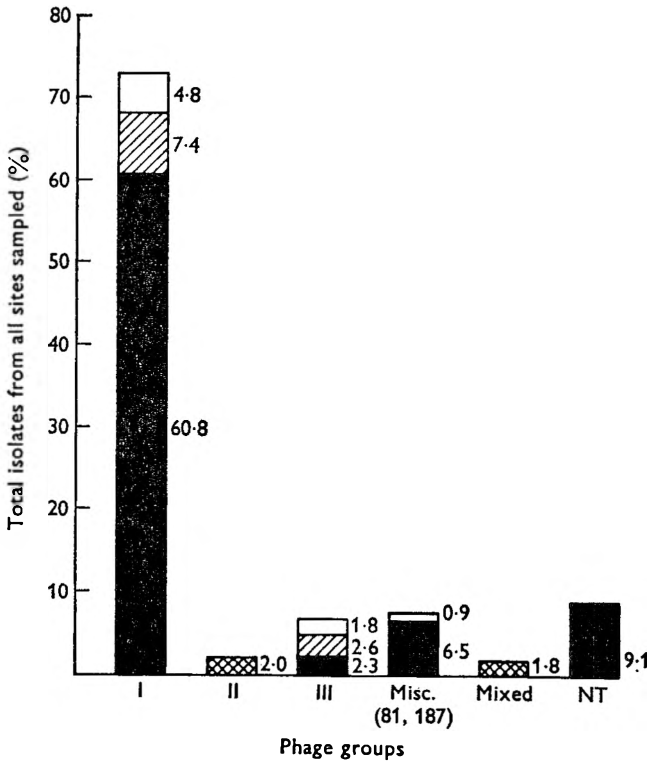

Fig. 3. Infants. Phage groups and antibiotic sensitivity patterns of *Staphylococcus pyogenes* strains. ■ Category A, strains sensitive to chloramphenicol, erythromycin and methicillin, and resistant to penicillin, streptomycin and tetracycline; ▨ category B, strains sensitive to all antibiotics except penicillin; □ category C, strains possessing other sensitivity patterns (majority multiple-sensitive); ▩ mixture of foregoing sensitivity patterns.

Phage typing of Staphylococcus pyogenes

Infants

A total of 567 strains from 182 infants were typed (171 strains from nose, 129 from post-nasal area, 267 from umbilicus). All three sites yielded almost the same incidence of the different phage groups, Fig. 3 representing the observations obtained from each site. Phage group I predominated, constituting 73·0% of all strains. Other investigators have found group III pre-eminent (Wallmark, 1953; Baldwin, Rheins, Sylvester & Shaffer, 1957; Gillespie *et al.* 1958; Hurst, 1960*a*). Hutchison & Bowman (1957) reported group III strains to account for 81·0% in a similar non-epidemic study.

Gould (1958) observed that when penicillin was in common use group III was the predominant phage group in ward air, and when penicillin was excluded groups I and II were most often encountered. Thus it may be that the use of the penicillinase-resistant penicillins together with other antimicrobial agents, i.e. the newer disinfectants, at the expense of penicillin, has resulted in the change to phage group I observed by us.

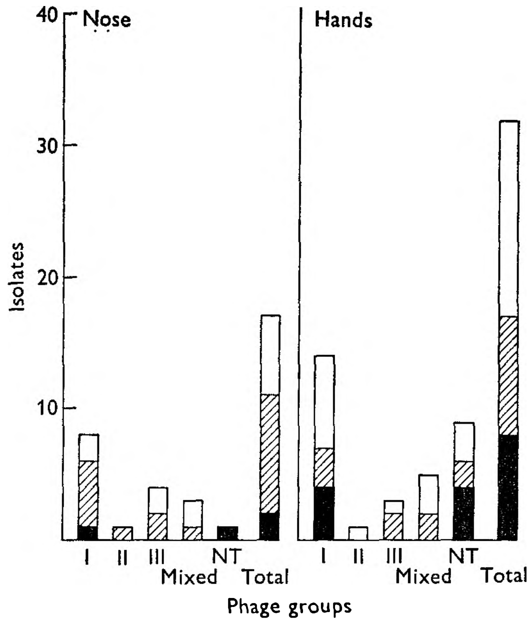

Fig. 4. Attending staff. Phage groups and antibiotic sensitivity patterns of *Staphylococcus pyogenes* strains. ■ Category A, strains sensitive to chloramphenicol, erythromycin and methicillin, and resistant to penicillin, streptomycin and tetracycline; ▨ category B, strains sensitive to all antibiotics except penicillin; □ category C, strains possessing other sensitivity patterns (majority multiple-sensitive).

Attendant staff

Forty-nine strains were typed, seventeen from the nose and thirty-two from the hands (Fig. 4). Group I composed most of the strains from the nose (47·0%) and the hands (43·7%). Baldwin *et al.* (1957) found group III to be the predominant nursery staff phage group (54·0%). However, our findings are supported by Poole (1960) and Barber (1961), who stated that strains obtained from nurses were usually of group I phage pattern.

Ward environment

Surfaces. Groups I and III were predominant, composing 37·5 and 31·2% respectively of typed strains (Fig. 5). This finding differs from that of Poole (1960), who reported no predominant phage group in forty samples from fomites. Yet other reports have shown group III pre-eminent in floor dust (Alder, Gillespie & Thompson, 1955; Hutchison & Bowman, 1957).

Air. Groups I and III were again the commonest found, constituting 43·1 and 33·3% respectively. Rountree & Barbour (1950) found group III most often in the air of a hospital nursery, as did Hurst (1960*a*).

Both surface and air strains appear to be showing a change in phage group, from group III of previous reports to approximately equal incidence of groups I and III in this study. This 'swing' towards group I parallels the results obtained with infants' strains, and the same interpretation is applicable, namely, that the introduction of the new penicillins is bringing about the disappearance of group III strains with their replacement by group I.

Antibiotic sensitivities of Staphylococcus pyogenes

Infants

All strains phage typed were tested for their sensitivity to antibiotics, and were placed into one of the three sensitivity categories already described. Fig. 3 illustrates the results obtained, and also shows the incidence of the three sensitivity categories within each of the phage groups. Group II and 'mixed' groups contained too few strains to justify separation into their component sensitivity categories. Separate analysis of the three sites sampled with regard to antibiotic patterns does not warrant description, Fig. 3 reproducing closely the results obtained from each of the sites.

Category A constituted 79·0% of all strains, this being the prevailing sensitivity throughout the hospital. Hutchison & Bowman (1957) found that 67·5% of their strains gave antibiotic-sensitivity patterns which correspond to our category C, only 14·5% belonging to our category A. Thus, most of their strains were penicillin-sensitive. Only 10·1% of our strains showed sensitivity to penicillin (this figure includes strains from group II and 'mixed' phage group), so we agree with Barber & Burston (1955), who found 11·0% of their strains sensitive.

When note is taken of the sensitivity pattern within each phage group category A was found to compose 83·3% of group I strains (Fig. 3). Group I strains possessing category A sensitivity pattern were, therefore, the colonizers of infants. It is of interest that this sensitivity category was found more often among group III strains by Barber & Burston, though Hurst (1960*b*) found group I to contain strains with this sensitivity pattern. Categories A and B, i.e. the penicillin-resistant staphylococci, compose 93·4% of group I strains and 73·1% of group III strains. These figures for penicillin resistance differ markedly from the results of Anderson & Williams (1956), who found 35% of group III strains and 17% of group I resistant to penicillin. However, our observation that penicillin resistance was commoner among group I strains is supported by Barber & Burston (1955).

Attendant staff

The most noticeable feature was the infrequency of Category A strains, only two of seventeen nasal strains and eight of thirty-two hand strains possessing this pattern (Fig. 4). Penicillin-resistant strains (categories A and B) constituted 64·7 % of nose staphylococci and 53·1 % of hand staphylococci; these results agreeing closely with previous reports (Ludlam, 1953; Barber & Burston, 1955; Hutchison & Bowman, 1957; Monro & Markham, 1958; Poole, 1960).

Among group I strains categories B and C were more frequently seen, which contrasts with the almost total predominance of category A in infants' group I strains.

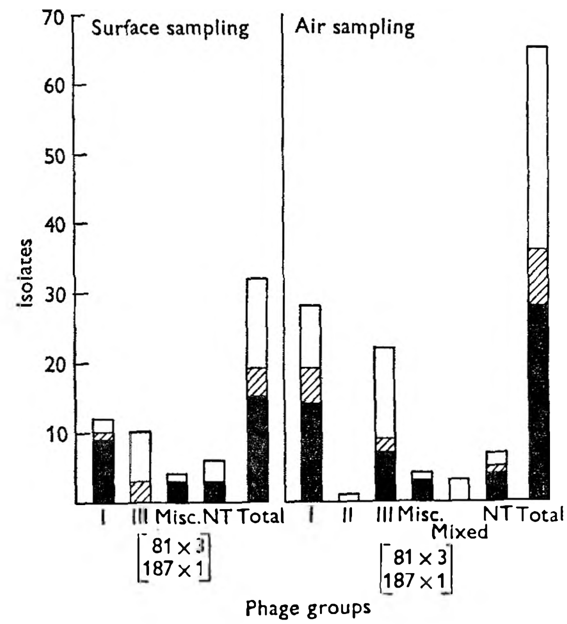

Fig. 5. Environment. Phage groups and antibiotic sensitivity patterns of *Staphylococcus pyogenes* strains. ■ Category A, strains sensitive to chloramphenicol, erythromycin and methicillin, and resistant to penicillin, streptomycin and tetracycline; ▨ category B, strains sensitive to all antibiotics except penicillin; □ category C, strains possessing other sensitivity patterns (majority multiple-sensitive).

Ward environment

Surfaces. Almost half the strains were of category A (46·9 %). (See Fig. 5.) Penicillin-resistant staphylococci accounted for 59·4 % of all strains, this being significantly less than the 87·5 % penicillin resistance quoted by Ludlam (1953) from forty-eight dust strains. This is probably due to the reduced use of benzylpenicillin since the introduction of the new penicillins.

Category A pattern was more often seen in group I strains, and category C in group III.

Air. The sensitivity patterns were very similar to those found among surface strains, category A making up 43·1 % and penicillin-resistant strains 55·4 % (Fig. 5).

Within the phage groups category A sensitivity pattern was linked with group I, and category C with groups I and III.

Clinical infection

During the period of this investigation, 247 infants were admitted to the nurseries. Of these, 5 died and 16 were discharged home or transferred to other units within 48 hr. of delivery and were excluded from the study. Of the remaining 226 infants 40 showed sufficient abnormality to justify bacteriological investigation involving 54 specimens (Table 4). Swabs of conjunctival discharge were taken from 18 infants and local antibiotic treatment was given to 16 of these. Specimens were of stool in 14 cases and no pathogenic organisms were isolated from 12 of these. From the remaining 22 specimens, organisms were cultured from 20. The organisms isolated from all specimens are shown in Table 4. Of the 36 organisms isolated, 16 were *S. pyogenes* and these came from 15 infants. The antibiotic sensitivity pattern showed that 14 belonged to category A, 1 to category B and 1 to category C. Phage typing was not carried out routinely on these strains. Seven of the thirty-six organisms isolated were Gram-negative bacilli and were isolated from the eye (2), urine (2), umbilicus (1), throat (1), skin (1).

Table 4. *Organisms isolated from cases of clinical infection*

Organism	Total	Eye	Skin	Umbilicus	Mouth	Nose and throat	Urine	Stool	Gastric washing
Staphylococcus pyogenes	16	8	4	2	—	—	—	2	—
Staphylococcus albus	5	4	—	—	—	1	—	—	—
E. coli	2	—	1	—	—	—	1	—	—
Proteus spp.	1	—	—	—	—	—	1	—	—
Ps. pyocyanea	1	—	—	1	—	—	—	—	—
Paracolon bacilli	3	2	—	—	—	1	—	—	—
Streptococcus pyogenes	3	—	—	2	—	1	—	—	—
Pneumococcus	1	1	—	—	—	—	—	—	—
Candida spp.	4	—	—	—	3	—	—	—	1
None	18	3	—	1	—	1	1	12	—
Total	54	18	5	6	3	4	3	14	1

Systemic antibiotic therapy was given to five infants. In two there was no bacteriological support to the clinical impression of infection. One of premature twins died at 18 days of age from bilateral adrenal haemorrhage, probably associated with septicaemia. Blood cultures were not carried out before death. *Staph. pyogenes* had been cultured from skin lesions and conjunctival discharge. This organism was also cultured from the twin whose blood culture was negative. She was also treated, and survived. The fifth infant was treated successfully for a respiratory infection, *Streptococcus pyogenes* having been cultured from the throat swab.

Thus, 21 of the 226 infants (9·3 %) showed clinical or bacteriological evidence of infection requiring local or systemic antibiotic therapy.

Ante-natal and post-natal wards

The patients in these wards were nose-swabbed on one occasion only. One isolation of *Staph. pyogenes* in significant numbers was made from eighteen ante-natal patients. This strain was non-typable and possessed category C pattern. This patient's infant subsequently yielded *S. pyogenes* from all three sites; these strains were all group I and of category A pattern.

Of twenty-six mothers who were examined after delivery two yielded *S. pyogenes* in significant numbers. One of these strains was of phage group I and category B sensitivity: her infant was not examined. The other mother yielded a group III strain of category B, but her infant was not a carrier.

No firm conclusions can be drawn from these findings in view of the small survey. Perhaps all that may be said is that there did not appear to be any transference of staphylococci between the mother and her infant.

No Gram-negative bacilli were isolated from any of these patients.

DISCUSSION

This combined laboratory and clinical study was undertaken to provide an indication of the behaviour of *Staphylococcus pyogenes* and Gram-negative bacilli in a hospital nursery not employing any specific antiseptic or aseptic methods (other than those described). Although the role of the staphylococcus in a nursery has been investigated extensively (mostly between 1942 and 1960), comparatively little attention has been devoted to the presence of the Gram-negative bacilli. We only know of one similar investigation which has examined the rate of colonization of infants with these bacteria, and which has also included the measure of their presence in the ward staff and in the ward environment (Shallard & Williams, 1965, 1966). However, these workers only swabbed infants at weekly intervals and were thus unable to show the day-by-day gradual acquisition of bacteria at the usual sites of carriage. Also no measure was given of separate nose and hand carriage of Gram-negative organisms by the staff. With the continuing use of antiseptics in the nursery, almost all of which are active primarily against the Gram-positive flora, the analysis of the Gram-negative bacilli becomes increasingly important.

Clinical sepsis

The pattern of clinical sepsis was that commonly found at the time of the trial. Surface infection of the eyes, skin and umbilicus accounts for the majority of the lesions, with *S. pyogenes* the predominant organism. The antibiotic sensitivity pattern, with fourteen of sixteen staphylococci belonging to category A, is in keeping with the organisms grown from the sampling of all infants but differs from the pattern found in the hands and noses of the attendant staff. Satisfactory bacteriological proof of infection as the cause of death of the premature infant who died from adrenal haemorrhage is lacking but with this probable exception there were no serious infections. Gram-negative organisms did not give rise to any infections of sufficient severity to justify systemic antibiotic therapy. This contrasts with the present position in the same nursery where Gram-negative infections are now the chief indication for systemic antibiotic therapy (Keay, Syme & Barnes, 1967).

Routes of spread of Staphylococcus pyogenes

Figure 6 depicts the possible routes by which we believe infection is transmitted in a hospital nursery. For each of the five main aspects of the nursery (air, ward surfaces, staff hands, staff noses, infants' carriage sites) we have formulated a pattern combining the predominant phage group isolated with the most fre-

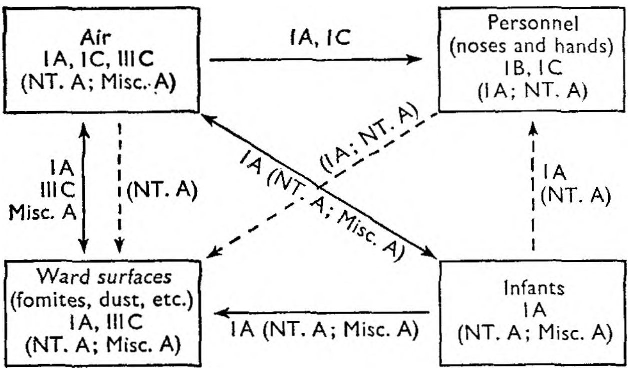

Fig. 6. Routes of transmission of *Staphylococcus pyogenes*. IA, IIIC, etc., denote strains of phage group I, sensitivity category A; phage group III, category C, etc. NT. A = non-typable strain, category A. Misc. = miscellaneous phage group. Strains in parentheses are regarded as secondary colonizers. Continuous line = probable route of transmission; broken line = possible but not proven route.

quently occurring antibiotic sensitivity category. This pattern is presented as IA, IIIC, etc. Less frequently encountered patterns are shown in parentheses. The ward air, for example, contained staphylococci of IA, IC and IIIC patterns predominantly, and less frequently NT.A (non-typable category A), and miscellaneous A.

We believe the ward air to be the primary *depot* of nursery staphylococci. Transmission of aerial staphylococci occurred to both infants and the noses of attending personnel. The infants became colonized primarily with IA strains (Fig. 6). The aerial staphylococci which colonized the nose of the attending personnel possessed different patterns, IA strains being very infrequent. In summary, the infants and attending staff 'abstract' different staphylococci from the air as their dominant colonizing strains. There is no transfer of staphylococci from the noses of the attending staff to infants.

The air is also a *depot* for direct transmission of staphylococci to ward surfaces. Both IA and IIIC patterns were deposited from the air on to the floors and other ward objects including bedclothing.

The infants, once colonized, become a sub-depot for transmission of *some* of their strains (I A, NT.A) to the hands of the attending staff and thence to ward objects. It was observed that *S. pyogenes* of the miscellanous phage group were only isolated from the air, infants and ward surfaces (Fig. 6) and not at all from the attending staff. Similarly III A strains were only isolated from the air and the infants. The hands of the nurses cannot, therefore, be implicated as an important route of spread of staphylococci from infant to infant.

Ward surfaces and infants are, therefore, contaminated primarily from the air. The question thus arises: from what *source* do the aerial staphylococci come? We believe that there are two sources, one being one or more of the infants in the ward, those infants being active 'dispersers', and the other source being the ward surfaces. Those infants who are active 'dispersers' may be infants normally colonized in the nose but who have been converted into 'dispersers' or 'spreaders' following therapy with tetracycline, kanamycin or the penicillinase-resistant penicillins (Ehrenkranz, 1965). The staphylococci are dispersed into the air around the infant, and also to the cot bedclothing. Thus the second source becomes obvious, namely, the ward surfaces, which include the blankets and the floor. There develops a constant interchange of bacteria between the ward surfaces and the air, this being readily brought about by such routine ward activities as cot-making and the movement of cots and other objects during ward cleaning. The importance of the presence of the ward personnel in disturbing floor dust cannot be underestimated. However, the personnel play no part in *direct* transmission of staphylococci, they are merely 'onlookers at the cycle, infant → ward surfaces → air → infant', as depicted in Fig. 6.

Efforts designed to reduce the load of aerial bacteria should result in a lowering of infant colonization rates and ward surface contamination, and this has been shown to occur following the use of methicillin aerosols in wards (Elek & Fleming, 1960). An alternative method of reducing the aerial staphylococci is to treat all the infants with an antiseptic effective against these bacteria and so minimize the consequences of infant 'spreaders' in the population. This latter method has been the subject of many trials, with varying degrees of success (Baldwin *et al.* 1957; Gillespie *et al.* 1958; Gluck & Wood, 1961).

Ecology of the Gram-negative bacilli

Nasal carriage of these bacteria was minimal, despite their not inconsiderable presence in the ward air. Such bacteria are, therefore, poor colonizers of the healthy infant's, and adult's, upper respiratory tract.

Gram-negative organisms colonizing the umbilical area of the infants had, almost certainly, resulted from spread from the infants' peri-anal area. We did not carry out a detailed examination of the umbilical flora, but it would be interesting to know the extent of the relationship which exists between the umbilical flora of the infant and its faecal flora. Perhaps comparative serotyping of *Esch. coli* strains from both the infant's umbilicus and its faeces would prove of value.

We propose that the transmission of Gram-negative bacteria in the hospital nursery parallels that of *S. pyogenes*, except that, (*a*) little, or no, nasal colonization by aerial Gram-negative bacilli occurs, and, (*b*) the infants' Gram-negative umbilical flora is of endogenous and not aerial origin. The air is thus the *depot* and the infants the *source* of these organisms. The nurses' hands convey some of the infants' Gram-negative flora to ward surfaces, but cannot be held responsible for infant to infant spread. The ward surfaces are further contaminated with bacteria sedimenting from the air. This latter mode of transmission also operates in reverse, there being a constant interchange between ward surfaces and the air, as we have already described for staphylococci.

SUMMARY

Two hundred and twenty-two infants and fifty-one staff were examined for carriage of *Staphylococcus pyogenes* and Gram-negative bacilli, and the presence of these bacteria in the ward environment was also investigated. Staphylococci were phage-typed and tested for sensitivity to antibiotics. Assessment of clinical infection caused by these bacteria was made.

Infant carriage of *Staphylococcus pyogenes* was maximal on the sixth day, 73·1 % of umbilical swabs yielding significant growth. Carriage of Gram-negative bacilli was maximal on the fifth day, 31·7 % of umbilical swabs being positive. Where special nursing was enforced, i.e. premature baby unit, isolation of Gram-negative bacilli was very infrequent.

The low nasal carriage of staphylococci seen in infants and nursing staff is attributed to traces of penicillinase-resistant penicillins in the ward air.

Staphylococcus pyogenes was recovered from 23 % of ward surfaces, and constituted 0·7 % of the air-borne flora. In contrast, Gram-negative bacilli were recovered from 4·7 % of surfaces, and composed only 0·15 % of the air-borne flora.

Phage group I constituted 73·0, 44·9 and 41·2 % of strains from infants, staff and ward environment respectively.

Antibiograms were grouped into three categories, A, B and C. Category A (sensitive to chloramphenicol, erythromycin and methicillin but penicillin-resistant) composed 79·0 % of infants' strains, being found mostly in group I, but was rare among staff strains.

Of 54 specimens taken from 40 infants showing evidence of infection, *Staphylococcus pyogenes* was isolated from 16, of which 14 were category A. Gram-negative bacilli were isolated 7 times. Only 21 infants required antibiotic therapy (9·3 %) and none were infected with Gram-negative bacilli—this contrasts with the frequency of these bacilli in nursery infection today.

Ward air is considered to be the main *depot* of *Staphylococcus pyogenes*. Infant 'dispersers' are probably the principal *source* of the air-borne staphylococci. Infants and staff abstract from the air those staphylococci which become their colonizing strains. No transfer is thought to occur from the staff to the infants. Transmission of Gram-negative bacilli is similar in that the infants are the principal source and the air the main *depot* of these bacteria. Nasal colonization by Gram-negative bacilli is low, this is possibly due to poor colonizing properties. Infant umbilical carriage almost certainly results from endogenous faecal spread.

We are grateful to Dr J. C. Gould, Director, Central Microbiological Laboratories, Edinburgh, for his continued interest and advice, and to Mr J. C. Ferguson and Mrs M. M. Winton for skilled technical assistance. This study would not have been completed without the co-operation of Sisters McCartney and Struthers and their nursing staff.

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Langat virus encephalitis in mice

I. The effect of the administration of specific antiserum

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Webb & Smith (1966) suggested that the clinical manifestations and histological abnormalities of virus diseases of the central nervous system (CNS) may be due not only to nerve cell destruction by virus multiplication but also to some kind of virus (antigen)-antibody reaction. The main reasons for this hypothesis were: (*a*) that the CNS disturbance almost invariably takes place after the end of the viraemia when antibody is increasing in the blood; (*b*) that only a very small percentage of all those infected actually develop clinical encephalitis, suggesting some individual response by the infected person which predisposes towards CNS involvement; and (*c*) that in immune tolerance experiments with lymphocytic choriomeningitis virus (Weigand & Hotchin, 1961) which invades the CNS, no disturbance occurs when an immunological response is absent.

The experiments reported here show that although administration of antibody before infection can protect mice from encephalitis, antibody given on certain days after infection can aggravate the disease. A second paper describes experiments which show how depression of the immunological mechanisms by irradiation affects the development of encephalitis in infected mice. Langat virus is particularly suitable for these experiments as it causes clinical encephalitis in only a small proportion of mice if given intraperitoneally (i.p.); thus it simulates to some extent the behaviour of similar viruses in humans.

MATERIALS AND METHODS

Langat virus

The TP21 strain of Langat virus was used in its seventh mouse passage as a 10% mouse brain suspension in bovine albumen phosphate saline (BAPS). Virus titrations were performed by diluting blood or mouse brain suspensions in 10-fold steps in BAPS and inoculating volumes of 0·03 ml. intracerebrally (i.c.) or 0·1–0·2 ml. i.p. in mice 3–4 weeks old. Blood samples for viraemia estimations were obtained from the orbital plexus using heparin as an anticoagulant. In several experiments half a brain was preserved for histological examination and half titrated for virus. Virus titres were calculated by the method of Reed & Muench (1938).

Mice

The mice used were of the Swiss A_2G strain of either sex, received from the Glaxo Research Farm, Harefield, Middlesex.

Antibody preparations

Human ascitic fluid samples were taken before and after the infections from a patient with carcinoma of the ovary who had been treated first with Langat virus and then with the closely related Kyasanur Forest disease virus (Webb, Wetherley-Mein, Smith & McMahon, 1966). The post-infection ascitic fluid used in these experiments was taken following the second infection. This fluid had a neutralizing index of at least 1000 and a haemagglutinin-inhibiting (HI) titre of 1/320 to Langat virus. Other human sera taken before and after Langat infections were used with similar results.

Swiss A_2G mouse serum was obtained before and after repeated i.p. inoculations of Langat virus. This serum had a neutralizing index of 630 and an HI titre of 1/40. All antibody preparations used were bacteriologically sterile.

Experimental

In the experiments the virus was given i.p. in 0·1–0·2 ml. quantities in doses ranging from 50 to 1,000,000 ICLD 50. The antibody preparations were given in doses of 0·1–0·2 ml. i.p., 0·1 ml. intravenously (i.v.) into the tail vein or 0·03 ml. i.c. For intracerebral or intravenous inoculation ether anaesthesia was used. In every experiment where an antibody preparation was given, a control group of mice was treated similarly with comparable material without antibody. None of the fluids were toxic to mice when inoculated alone. All mice were observed for at least 3 weeks and often for 4 weeks. In experiments where a sufficient number of mice survived, evidence of infection in them was established either by testing their sera for HI antibody, or (as this titre was low in many mice previously given antiserum early in the viraemia) they were challenged i.c. with 100–1000 ICLD 50 of virus. Paralysis rates rather than mortality rates are reported in the Tables because some paralysed mice survived to the end of the observation period. Most mice, however, had died by the twenty-first day.

Antibody studies

Haemagglutinin-inhibiting antibody levels were determined by the method of Clarke & Casals (1958) as modified by Sever (1962) and others. The heparin contained in the mouse plasmas was found to cause non-specific haemagglutinin-inhibition against Semliki Forest virus but not Langat virus. It was removed by treatment with protamine sulphate during acetone extraction (Holden, Muth & Skinner, 1966) without loss of antibody. The plasma extracts were tested against eight haemagglutinin units of Langat virus and of Semliki Forest virus as a control.

Histology

Brains and spinal cords were fixed in 10% formol saline and embedded in paraffin wax. Sections of at least five levels of the brain and three levels of the spinal cord were cut at 5 μ and stained with haematoxylin and eosin. All brains were fresh when fixed and any mice found dead were discarded. The distribution and severity of lesions seen in brain sections were graded as follows (modified after Berge *et al.* 1961):

0 (negative): No evidence of perivascular cuffing or increase in glial cells, normal mouse brain.

1: Mild perivascular cuffing and/or slight focal increase in glial cells.

2: Definite perivascular cuffing without distension of the Virchow–Robin spaces by multilayered cells and/or definite glial nodules without disturbance of histological architecture.

3: Marked perivascular cuffing and moderate increase in cellularity of glial nodules, with in addition more diffuse glial proliferation. Slight disturbance of background architecture and minimal neuronal damage present.

4: Marked perivascular cuffing with considerable proliferation of glial elements with alteration of ground substance often with vacuolization. Obvious neuronal destruction present.

RESULTS

Infections without administration of antibody

Many preliminary control experiments were carried out in which mice were killed on various days after infection either i.c. and i.p., and their brains and spinal cords examined histologically.

Intracerebral inoculation

There was a diffuse encephalomyelitis with a fairly rapid progression from the appearance of the first lesions as early as 5 days after infection until death on the seventh to ninth day. There was well defined mononuclear cell infiltration of the leptomeninges and of the perivascular spaces with a variable amount of migration of these cells into the surrounding brain substance. There were areas where proliferation of all the neuroglial elements, astrocytes, oligodendroglia and microglia, had occurred. These changes were seen at all levels of the brain and spinal cord. Neuronal changes, which were prominent in those mice which were clinically paralysed and ill, consisted of a patchy necrosis of all layers of the cerebral cortex of the hippocampus and caudate nucleus-putamen. The majority of the affected cells were shrunken and eosinophilic with pyknosis or karyorrhexis of the nuclei. Degeneration of the Purkinje cells of the cerebellum and of the ventral horn cells of the spinal cord was sometimes seen. The majority of the mice which were ill when killed had lesions of grade 4.

Intraperitoneal inoculation

The encephalomyelitis when this did occur was essentially similar. However, the progression of lesions was slower, the first changes were seen as early as the fifth day, yet advanced encephalomyelitis was often seen as late as the fifteenth day. There was also much greater variability in the intensity of the lesions. Although lesions were seen in all mice, in many these consisted only of scattered glial nodules and perivascular cuffs. The lesions in the most severely affected brains were of a characteristic distribution: the olfactory lobes and tracts, the cerebral cortex, the corpus striatum, the cerebellum and the grey matter of the spinal cord were constantly involved but the lesions were much less diffuse than following i.c. inoculation

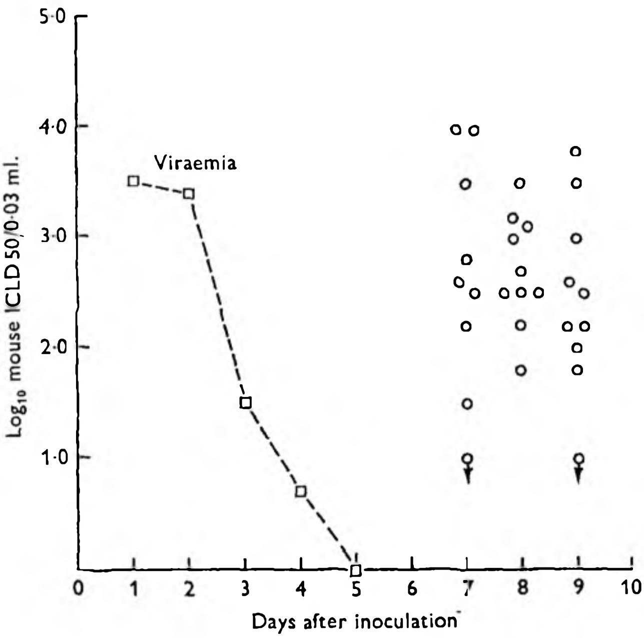

Fig. 1. Viraemia and virus titres in brain after intraperitoneal inoculation of 10,000 ICLD 50 of Langat virus on day 0. □ Viraemia (four bloods pooled each day). ○ Individual brain virus titres on days 7, 8 and 9.

Infections with administration of antibody

These experiments were designed to demonstrate the effect of antibody given 1–2 hr. prior to inoculation of virus, and on each post-inoculation day up to the sixth, which was the day before the first mice showed paralysis. As many as fifty mice were used in each group in some experiments so that both survival and histological studies could be done simultaneously. There were never less than eighteen mice in any group. Figure 1 shows the viraemia pattern in mice following i.p. infection. Mice with no evidence of clinical encephalitis were found nevertheless to have both histological encephalitis and high brain virus titres. In this experiment only nine of the thirty mice observed for symptoms had an encephalitic illness, and five of them died. However, all the brains of the similarly inoculated mice taken on days 7, 8 and 9, had incontrovertible evidence of encephalitis and all except two showed more than ten ICLD 50 virus per 0·03 ml. of a 10% suspension. This was at a time when viraemia had ceased at least 48 hr. earlier. The higher the virus dose the earlier the viraemia started. Older mice tended to have viraemia a little later than the younger mice, but viraemia had ceased before the sixth day in all.

Table 1. *Comparison of the viraemia following i.p. inoculation with* 10,400 *ICLD* 50 *of Langat virus in a control group and in experimental groups, one of which was given* 0·2 *ml. i.p. of a* 1/4 *dilution of pre- or post-infection human ascitic fluid on each of the first* 5 *days after inoculation*

Pre-infection ascitic fluid given on post-inoculation day	Virus titre in blood on post-inoculation day						
	1	2	3	4	5	6	7
1	.	2·5*	1·5	0·8	1·3	0·3	+
2	.	.	1·5	1·1	1·5	0	0
3	.	.	.	1·5	1·5	+	0
4	.	.	.	.	0·7	0	0
5	.	.	.	.	.	+	0
Controls for both groups	2·0	2·2	2·0	1·2	1·3	0	0
Post-infection ascitic fluid given on post-inoculation day							
1	.	+	+	0	0	0	0
2	.	.	0	0	+	+	0
3	.	.	.	+	+	0	0
4	.	.	.	.	0	0	0
5	.	.	.	.	.	0	0

* Virus titres measured in $\log_{10}$ mouse ICLD 50/0·03 ml.
\+ = Virus just detectable when neat blood was inoculated.
0 = No virus detectable when neat blood was inoculated.

Table 2. *Comparison of the effects of* 0·1 *ml. of undiluted pre- and post-infection human ascitic fluid given i.p. to a group of* 4–6 *weeks old mice on each of the first* 6 *days after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus*

	Proportion of mice paralysed after	
Ascitic fluid given	Post-infection ascitic fluid	Pre-infection ascitic fluid
Before infection	0/25	7/25 (28)
(Days after)		
1	16/24 (67)	9/24 (37)
2	17/24 (71)	5/24 (21)
3	9/24 (37)	8/24 (33)
4	8/24 (33)	5/24 (21)
5	2/24 (8)	5/24 (21)
6	4/24 (17)	6/24 (25)

Figures in parentheses indicate percentages.

Table 1 shows the viraemia of mice after administration of a 1 in 4 dilution of pre- or post-infection human ascitic fluid to a group of mice on each day after inoculation of virus. A control group given virus only was included. On each day, two mice were bled and killed, their bloods pooled and titrated for virus, and their brains kept for histology. The pre-infection fluid had no significant effect on viraemia. The post-infection fluid quite clearly depressed the viraemia, though a trace was detectable occasionally. Histological lesions were first seen on the sixth day in all groups. There was a marginal reduction in the histological evidence of encephalitis when post-infection fluid was administered on days 1–4 as compared to pre-infection fluid. When post-infection fluid was administered on the fifth day, a slight increase in the number of lesions was seen as compared to mice given pre-infection fluid.

Table 2 shows the paralysis rates when human post-infection fluid was given i.p. on the specified days compared to pre-infection fluid controls. Here, the post-infection fluid clearly provided complete protection when it was given before virus inoculation, whereas, when it was given on either of the first 2 days, paralysis was significantly more frequent.

Table 3. *Comparison of the effects of* 0·1 *ml. of undiluted pre- and post-infection human ascitic fluid given i.v. to a group of* 4–6 *weeks old mice on each of the first* 5 *days after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus*

Ascitic fluid given after infection (day)	Proportion of mice paralysed after	
	Post-infection ascitic fluid	Pre-infection ascitic fluid
1	9/19 (47)	2/18 (11)
2	13/20 (65)	5/20 (25)
3	9/20 (45)	6/20 (30)
4	2/20 (10)	4/20 (20)
5	1/20 (5)	1/20 (5)

Figures in parentheses indicate percentages.

Table 4. *Comparison of the effects of* 0·1 *ml. of pre- and post-infection human ascitic fluid, normal mouse serum and mouse antiserum given i.v. to* 6–8 *weeks old mice on the third day after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus*

Proportion of mice with paralysis after			
Post-infection ascitic fluid	Pre-infection ascitic fluid	Mouse antiserum	Normal mouse serum
17/28 (61)	4/29 (14)	14/23 (61)	2/24 (8)

Figures in parentheses indicate percentages.

Table 3 shows the paralysis rate in the mice when human post-infection fluid was given intravenously on the specified days compared to pre-infection controls. Once again, a significantly increased paralysis rate occurred when antibody was given 1 or 2 days after virus. Both these Tables suggest a decreased paralysis rate when post-infection fluid is given particularly on days 5–6 (after the height of the viraemia). The possible reasons for this are discussed at the end of the following paper.

Table 4 compares the paralysis rates when mouse normal serum or antiserum, or human pre- or post-infection ascitic fluid was given i.p. on the third day. This shows that the difference in paralysis rate when antibody is given on the third day can be as great as seen in the previous experiments when it was given on the first 2 days.

In all these experiments paralysis in control mice and in those given pre-infection fluid was first observed between the sixth and thirteenth day after infection (most commonly on the eighth day), whereas in the mice given post-infection fluid on any day up to the fifth day, the earliest onset of paralysis was on the thirteenth day

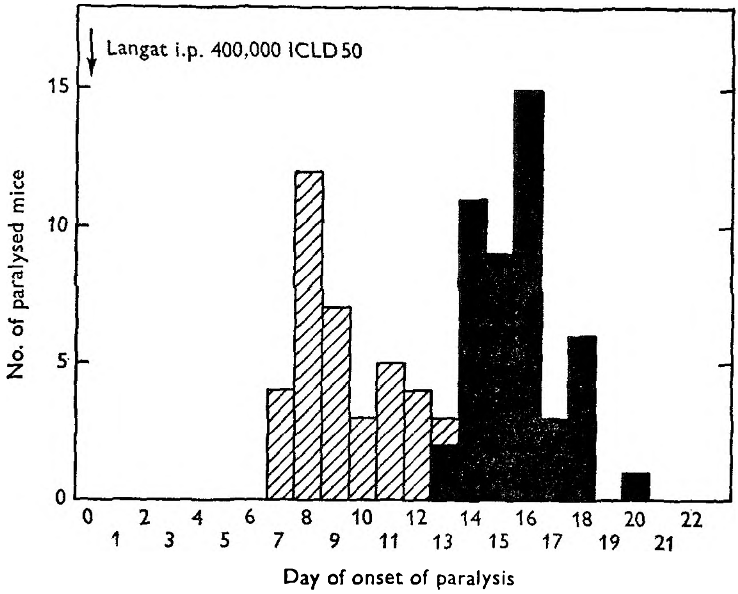

Fig. 2. Day of onset of paralysis after intraperitoneal inoculation of 400,000 ICLD 50 of Langat virus only (controls) and after pre- and post-infection ascitic fluid on days 1–3. ▨ Mice receiving pre-infection ascitic fluid and control mice. ■ Mice receiving post-infection ascitic fluid.

after infection, but could be delayed to the twentieth day (most commonly on the sixteenth day) (Fig. 2). Thus, the antiserum delayed the onset of paralysis by about 7 days, and increased the proportion affected compared to those mice given virus only and mice given virus followed by normal serum.

In the experiment reported on Table 4, up to four mice were taken for histology from each of the four groups (human post- and pre-infection fluid and mouse antiserum and normal serum) on at least 3 days between the seventh and fourteenth days after infection. Eight mice from the group which had received mouse antiserum were taken after the onset of clinical paralysis 17–21 days after infection, each being matched with a mouse from the non-immune group (none of which showed evidence of paralysis). Finally, ten clinically healthy mice were taken from each group 21–28 days after infection, and after bleeding were killed for histological examination. There were separate groups for histological examination quite distinct from those for observation. The results fall into three categories:

Category 1. *Mice examined before paralysis* (7–14 *days*)

Three or four mice were selected at random from each group on each day, thus some of these mice would have subsequently become paralysed had they been permitted to survive. Table 5 shows that there is a clear difference between the groups which received human post- and pre-infection ascitic fluid: in the latter group mild lesions were first seen in 2/4 mice on the seventh day, and there was a steady increase in intensity of the lesions up to the fourteenth day, all mice being affected; in contrast, only one of the former group on the seventh or ninth day showed any evidence of encephalitis (graded 1) and only 2/4 and 2/3 taken on the eleventh and fourteenth days respectively were affected.

Table 5. *Comparative histology in groups of four mice given* 0·1 *ml. i.v. of pre- or post-infection ascitic fluid* 3 *days after infection i.p. with* 400,000 *ICLD* 50 *of Langat virus, and killed* 7–14 *days after infection*

Days after infection with virus	Human ascitic fluid							
	Post-infection				Pre-infection			
7	0	0	0	1	0	0	1	1
9	0	0	0	0	1	1	1	2
11	0	0	1	4	1	2	3	3
14	0	2	4	—	3	3	3	4

No comparable histological differences in time of onset were found between the two groups which had received mouse serum. However, the mouse antiserum had a much lower titre than the human post-infection ascitic fluid used.

Category 2. *Mice examined after the onset of paralysis* (17–21 *days*)

These were all taken from the group which had received mouse antiserum and were compared with mice which had received normal mouse serum and were not paralysed. All the paralysed mice showed extensive changes (graded 4, one 3): particularly in the hind brain and spinal cord, whereas all the non-immune group showed mild changes (1 or 2, one mouse 3). This latter finding was unremarkable since there were no new cases of illness as late as the seventeenth day in the mice in this group which were kept for observation and thus severe changes would not be expected. Consequently, it was decided to compare the histological changes in the paralysed group with those in mice from previous experiments which had received a comparable dose of virus and which had been paralysed for a similar period before being killed. They had received no serum or treatment of any kind and had become paralysed 7–10 days after infection. There was no obvious difference between the different members of this group, and all had lesions of grade 3 or 4, maximal in the spinal cord. There were, however, several differences in the distribution and emphasis of lesions between this control group and the paralysed group which had received antiserum, although the overall grades were closely similar in both groups. Lesions in the cerebellum were more frequent and intense in the group which had received antiserum; in particular there was more perivascular cuffing in the central white matter, and the thickness of the cuffs tended to be greater. Lesions were also of greater intensity in the spinal cord with, again, a slight increase in number and thickness of perivascular cuffs, together with an increase in the amount of reactive glial hyperplasia and neuronophagia. In contrast there were fewer lesions elsewhere in the CNS than in the control group. Mice given human ascitic fluid were not examined at this stage.

Table 6. *The comparative histology and HI titre in mice given pre- and post-infection human ascitic fluid and surviving* 21 *days*

	Human ascitic fluid			
	Post-infection		Pre-infection	
Day after inoculation	Grade	HI	Grade	HI
21	0	< 10	1	40
	0	< 10	2	⩾ 160
	2	20	3	80
	2	40	2	80
	0	< 10	1	80
	2	10	2	40
22	0	< 10	2	⩾ 160
	3	20	2	80
	2	10	1	⩾ 160
	1	< 10	2	⩾ 160
	2	20	1	80
23	2*	20	1	⩾ 160
	3*	20	3	⩾ 160
	2*	20	1	80
	1*	10	1	⩾ 160

* = Paralysed mice.

Category 3. *Mice taken* 21–28 *days after infection*

These mice were selected because at this time new cases of illness had ceased to appear in the observation groups. In the human ascitic fluid groups (Table 6) four of the fifteen mice which had received post-infection fluid escaped lesions as compared to none of the twenty which received pre-infection fluid. Apart from this there were no obvious differences histologically between these groups. All these mice were bled before they were killed and their HI antibody was measured. The values were significantly lower (geometric mean eight-fold lower) in the mice which had been given human post-infection fluid. Notably four of the five mice without HI antibody were the four mice without histological lesions. This may have been due to failure to infect. It is also of interest that the four mice killed on the twenty-third day were all paralysed and it can be seen that they too have HI antibody titres in the lower range. None of these distinctions were observed in the groups which had been given mouse serum.

Table 7 shows the average time of death and paralysis rate of mice given virus i.p. and post- or pre-infection ascitic fluid i.c. on the specified days. Even 0·03 ml. of post-infection fluid given i.c. before inoculation of virus completely protected the mice and, in contrast to the previous experiments, intracerebral post-infection fluid on the first three post-inoculation days also provided some immunity. Furthermore there was a marked increase in death rate compared with the other experiments with pre-infection fluid. This was almost certainly due to the procedures of needling of the brain and thereby introducing virus directly into it. Our experience has shown that i.c. inoculation given just before or at the time of the viraemia greatly increases the mortality of the mice: not immediately following the inoculation but as a very greatly increased rate of paralysis and death at the normal time expected. It seems probable that the protection produced by inoculating the brain with antiserum at the height of the viraemia is because the secondary virus cycle in the brain is suppressed.

Table 7. *Comparison of the effects of* 0·03 *ml. of undiluted pre- and post-infection human ascitic fluid given i.c. to groups of twenty-five mice before and on each of the first* 5 *days after infection i.p. with approximately* 400,000 *ICLD* 50 *of Langat virus*

	Post-infection ascitic fluid		Pre-infection ascitic fluid	
Serum given	ADD*	Paralysed	ADD	Paralysed
Before infection		0/25	9·5	19/25 (76)
(days after)				
1	16·5	13/25 (52)	9·2	24/25 (96)
2	15·0	10/25 (40)	10·0	17/25 (68)
3	16·0	8/25 (32)	10·3	11/24 (46)
4	14·5	12/23 (52)	10·0	6/25 (24)
5	8·2	10/23 (43)	10·1	11/25 (44)

* Average day of death.
Figures in parentheses indicate percentages.

DISCUSSION

These experiments have clearly shown that when specific antiserum was given to mice on any of the first 3 days (when the viraemia would otherwise be at its highest), the viraemia was depressed, the development of antibody was depressed and possibly delayed and the incidence of encephalomyelitis was significantly increased. Thus, as postulated by Webb & Smith (1966), an immunological reaction probably plays some part in the pathogenesis of the clinical disturbance. It is also clear (Tables 2 and 7) that a small dose of specific antibody administered i.p. or i.c. before virus inoculation was sufficient to protect the animals completely even against large doses of virus. This is compatible with the accepted use of passively administered antibody in the prophylaxis of disease. The results of the experiments using human ascitic fluid cannot be explained on the basis that human antibody was becoming bound to virus perhaps at the surface of a CNS cell and that the host then rejected the antigen-antibody complex (as foreign protein) together with the cell: similar results were obtained when mouse antiserum (prepared in the same strain of mice) was used. Moreover, it is well known that it is very difficult to produce experimental allergic encephalitis in mice except in especially selected strains.

Histological examination of brains and spinal cords tended to confirm the other findings without making any positive contribution towards a solution. The finding that the onset of histological encephalitis is delayed and that the intensity of lesions between the seventh and fourteenth day (category 1) was less in animals which received human antibody than in those which received pre-infection fluid tends to support the clinical and virological results over the same period. Thus, one would expect a lower viraemia and delayed host antibody production to be associated with fewer and delayed CNS lesions. The absence of a similar histological difference between the two groups which received mouse antiserum and normal serum was probably due to the lower antibody titre of the mouse serum. Nevertheless, there was still delayed onset and an increased incidence of encephalitis. The mice which had been given mouse antiserum on the third day and were killed after the onset of paralysis (days 17–22, category 2) had more severe encephalitis and myelitis than controls (given pre-infection ascitic fluid) which were clinically healthy at this stage. Comparing the histology of the paralysed mice from this group with that of paralysed mice in control groups falling sick at the usual time, the same histological gradation was found. However, in the antibody group the lesions were concentrated in the hind brain and spinal cord, with fewer in the remainder of brain. These findings are however of doubtful significance in view of the differences in the character of the two groups of mice.

Amongst the mice of category 3 which had been given human ascitic fluid (i.e. those surviving mice taken on days 22–28 after new cases of paralysis had ceased to appear in the parallel observation group) it was of interest that four of the five mice without HI antibody were the four mice without histological lesions (Table 6). The post-infection fluid appeared to have protected these mice from infection. Thus, if these were eliminated the percentage of infected mice which developed paralysis of late onset would be even higher than indicated in Table 6, i.e. 17/24 (71 %).

Berge *et al.* (1961) performed similar experiments to those reported here using Venezuelan equine encephalomyelitis (VEE) virus in mice. They found that the administration of specific antiserum up to 24 hr. after virus was followed by the appearance of histological lesions at an earlier stage than in controls given normal serum. VEE virus has a shorter incubation period than Langat virus. However, these lesions remained mild and were associated with a high survival rate. The control mice which received normal serum all died and histological examination showed very severe CNS lesions just before death. Thus, our results differ from these in several ways: (*a*) VEE virus kills all mice when administered i.p. in sufficient dosage, unlike Langat virus, thus Berge *et al.* could not have found an increase in clinical disease; (*b*) they observed their mice for only 10 days, and may therefore have failed to observe the late onset of paralysis seen in our experiments. The following paper (Webb *et al.* 1968) contains a fuller discussion of the way in which the immunological response may affect the development of the encephalitis.

SUMMARY

1. When mice are infected intraperitoneally with Langat virus only a small proportion develop clinical encephalitis, but all mice have substantial titres of virus in the brain and also incontrovertible histological evidence of encephalitis.

2. When specific antibody is given intraperitoneally or intravenously to mice during the first 3 days after intraperitoneal infection with Langat virus, the viraemia (normally maximal during this period) is depressed, the production of antibody is depressed or delayed, and the incidence of clinical encephalomyelitis is increased significantly.

3. Specific antibody given intraperitoneally or intracerebrally before infection, protects the animals from encephalitis.

4. These findings are discussed in terms of the histology of the central nervous system of the affected mice.

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Langat virus encephalitis in mice

II. The effect of irradiation

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In view of the known suppressive action of irradiation on the immune response (Taliaferro, 1957) the following series of experiments have been carried out in an attempt to further evaluate the role of antigen-antibody reactions in the development of viral encephalomyelitis.

MATERIALS AND METHODS

These were as described in the previous paper (Webb, Wight, Platt & Smith, 1968). The same strain of mice was used but they were 22–25 days old in the majority of the experiments. Mice of ages ranging up to 8 weeks were used to obtain a base line for irradiation dosage. Since in these preliminary experiments a standard age seemed to be more important than a standard weight, mice 22–25 days old were used as their age could be more exactly estimated.

Antibody studies. IgM (19S) and IgG (7S) levels were measured by treating the mouse plasmas with 2-mercapto-ethanol (2-ME) after acetone extraction as follows. One volume was incubated for 2 hr. at room temperature with an equal amount of buffered saline containing a final concentration of 0·1 M 2-ME. Another volume was similarly incubated with an equal volume of normal saline. The 2-ME-treated extracts were then dialysed overnight in the cold against buffered saline containing 0·02 M iodoacetamide. Otherwise the method was as in the previous paper.

Irradiation was by gamma rays from ^{60}Cobalt sources. The experiments were conducted between June 1965 and May 1967. During this period the cobalt sources were renewed and in order to obtain uniformity all calculations are based on the activity of the various sources on the 1st January 1966, a decay factor based on a half life of 5·25 years being employed. The doses were checked under experimental conditions by a Baldwin–Farmer substandard dose meter with a Perspex build-up cap added to give electron equilibrium, bolus being added to simulate the body of a mouse.

Up to seven mice were irradiated at the same time. Each mouse was held in a plastic tube 3·1 cm. overall diameter, with a wall thickness of 0·2 cm., closed at either end by perforated rubber bungs. The mice were held in a jig on the central axis of a twin-headed cobalt unit so that they were in a single vertical plane with a tube of bolus above and below the top and bottom mouse respectively. The dose variation for the top and bottom mouse as compared with the dose received by the middle mouse was 95·5 %. Irradiation was given from both sources simultaneously, the beams being 24 × 24 cm. The dose rate was approximately 500 rads in 4·5 min.

Data from all our irradiation experiments have been plotted graphically and the irradiation LD 50 for 21-day-old mice, under our experimental conditions, was approximately 580 rads.

RESULTS

Table 1 shows how irradiation increased the sensitivity of the mice to peripheral infection with virus. Following intracerebral (i.c.) inoculation there was a higher virus titre in the irradiated animals compared with the controls. With intraperitoneal (i.p.) inoculation while all irradiated animals given dilutions from 10^{-3} to 10^{-8} died, only 15 of 36 controls over a similar dose range died (varying from 0 to 4 in each group of 6). As the i.p. inoculum given at each dilution was approximately 7 times as large as that given i.c., irradiation raised the i.p. virus titre

Table 1. *Titrations in control and irradiated mice* 3 *weeks old inoculated i.c. or i.p. with Langat virus.* 500 *rads were given* 24 *hr. before inoculation*

	Virus given i.c. 0·03 ml.				Virus given i.p. 0·2 ml.	
	Irradiation		Controls		Irradiation	Controls
Dilution of virus	Deaths	ADD*	Deaths	ADD	Deaths	Deaths
10^{-3}	—	—	—	—	6/6	4/6
10^{-4}	—	—	—	—	6/6	2/6
10^{-5}	—	—	—	—	6/6	2/6
10^{-6}	—	—	—	—	6/6	3/6
10^{-7}	5/5	10·0	5/5	9·6	6/6	4/6
10^{-8}	4/5	12·5	4/5	9·5	6/6	0/6
10^{-9}	3/5	12·3	0/5	—	—	—
10^{-10}	1/5	16·0	0/5	—	—	—
LD 50	$10^{-9.2}$	—	$10^{-8.4}$	—	$\geqslant 10^{-8.5}$	—

* Average day of death of mice.

approximately to the i.c. titre and gave it a clear end-point as compared with a very scattered one in the controls. This experiment also showed that, although the virus titre was higher in the irradiation group inoculated i.c., the average day of death (ADD) of the mice at the dilutions 10^{-7} and 10^{-8} was later than in the control group although the total mortality was identical. The control animals in these experiments died an unquestionable paralytic death, whereas the majority of the irradiated mice merely became hunched up with ruffled fur but no clinical evidence of paralysis was observed.

Table 2 compares the brain virus titres when approximately 100 ICLD 50 of virus was given i.c. either with or without preceding irradiation. Again, there was a significant prolongation of the ADD of the irradiated mice ($P < 0.01$). To make the experiment more complete a further five mice had been added to each group and treated in a similar manner except that they were killed on the seventh day after infection. Their brains were divided sagittally: one half was titrated for virus and the other half preserved for histological examination. Table 2 shows that although the titres of the brains from the irradiated group were, if anything, higher than those of the controls, the brains of the irradiated mice showed no histological changes, while the control brains all showed the characteristic changes of encephalitis.

Table 2. *The average day of death, brain virus titres and brain histology on the 7th day after inoculation in mice given* 500 *rads irradiation and then inoculated* 24 *hr. later with* 100 *ICLD* 50 *of Langat virus i.c., compared with mice given the virus only*

	Nos. in group	Deaths (%)	ADD in days	Brains, day 7: Mean virus titre ($\log_{10}$)	Brains, day 7: Histological encephalitis
Group 1. Irradiation and virus	14+5	100	10·6	6·8*	0/5
Group 2. Virus only	14+5	100	8·9	5·6†	5/5
Group 3. Irradiation only	7	0	—	—	0/7

* Range 6·3–7·2. † Range 3·7–6·5.

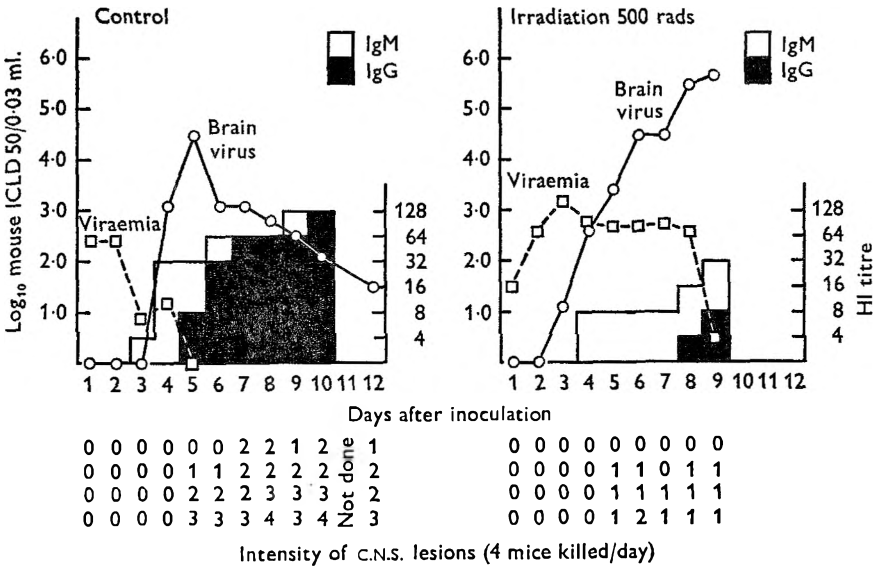

Fig. 1. Viraemia, brain virus, IgM and IgG titres and intensity of histological lesions following intraperitoneal inoculation of 1,000,000 ICLD 50 of Langat virus, 24 hr. after whole body irradiation with 500 rads.

Figure 1 depicts the results of an experiment in which approximately 1,000,000 ICLD 50 of virus was inoculated i.p. into control animals and into a group which had been irradiated with 500 rads 24 hr. previously. Following infection four mice were killed from each group on each day up to the tenth and on the twelfth day; all the irradiated group had died by the tenth day. The bloods and sagittal half brains of each of these groups of four mice were then pooled and titrated for virus. The remaining half brains were examined histologically, and the bloods tested for antibody. During the first 3 days the brain virus titre was either negligible or well below the blood virus titre. From the fourth day the brain virus titres exceeded the viraemia titre, but while in the control animals the brain virus reached its maximum on the fifth day and then subsided, in the irradiated mice the brain virus titre continued to rise and reached levels higher than even the maximum of the control mice. Figure 1 also shows the reciprocal serum antibody titres; there was little, if any, delay in the appearance of IgM antibody in irradiated mice although its titre may have been lower and it may have persisted longer. There was, however, a delay of about 3 days in the appearance of IgG antibody in irradiated mice and their overall antibody response was clearly diminished. In both cases, the viraemia began to fall with the appearance of detectable IgM antibody and ceased shortly after IgG became detectable.

A similar experiment in irradiated mice was performed using a much smaller virus dose (100 ICLD 50). The results were similar except that viraemia was not detected until the third day. The titre then remained at about 1000 ICLD 50 per 0·03 ml. until the ninth day, the one before death. There was a gradual rise in the brain virus titre from the fifth day until death as in the previous experiment.

Figure 1 also incorporates the histological findings. The inflammatory lesions of encephalitis were first seen in the control mice on the fifth day. The striking feature of all the brains of mice which had been previously irradiated was the almost total absence of histological encephalitis compared with the controls, though the brain virus titre was higher in the irradiated mice. The majority of these brains differed in no way from those of normal uninfected mice. However, neuronal changes, an infrequent finding in the control mice, were seen as often in the irradiated group. They were seen most commonly in the granular layers of the cerebral cortex and in the hippocampus and consisted of shrinkage and eosinophilia of the cytoplasm with pyknosis or karyorrhexis of the nuclei.

In an experiment (Table 2) where approximately 100 ICLD 50 was inoculated i.c. and the brains were taken on the seventh day, the findings were similar. The brains of the mice which had been irradiated showed no evidence of the expected inflammatory lesions of encephalomyelitis, while the brains of the controls showed typical encephalomyelitis. The brain virus titres tended to be higher in the irradiated mice, although the difference was not significant.

Table 3 shows the result of an i.p. titration in mice 3 weeks old which had received different doses of irradiation 24 hr. before inoculation. A dose of 300 rads or greater increased the sensitivity of the mice to the maximum when all mice in all dosage groups died. The change in behaviour was most marked between 50 and 100 rads.

To bring together these results with those in the previous paper (Webb *et al.* 1968) some experiments were performed in which irradiation was followed by administration of pre- and post-infection ascitic fluid. Table 4 shows the results of one such experiment. The administration of post-infection fluid i.v. on day 3 did not significantly alter the survival of mice which had been given virus i.c. However, in those which had been given virus i.p. two-thirds of the group survived and the remainder had a prolongation of survival time. Paralysis occurred in this group after the same interval as in mice which had been given virus and post-infection fluid but had not been irradiated. As expected, there was a 100% death rate in the group which had received virus i.p. followed by pre-infection fluid i.v. An extra number of mice in each i.p. virus group were kept for histological examination: nine mice from each were killed on the twelfth day. Of the mice which had

Table 3. *Proportion of* 3 *weeks old mice dying after i.p. inoculation of dilutions of Langat virus, given* 24 *hr. after the mice were subjected to different doses of irradiation*

Dilution of virus	Controls	Rads of irradiation 24 hr before virus 50	100	200	300	400	500
10^{-2}	6/6	—	—	—	—	—	—
10^{-3}	4/6	3/6	4/6	5/6	6/6	6/6	—
10^{-4}	2/6	0/6	3/6	6/6	6/6	6/6	6/6
10^{-5}	2/6	2/6	3/6	5/6	6/6	6/6	6/6
10^{-6}	3/6	2/6	6/6	5/6	6/6	6/6	6/6
10^{-7}	4/6	4/6	5/6	6/6	6/6	6/6	6/6
10^{-8}	0/4	2/6	4/6	5/6	6/6	6/6	6/6

Table 4. *Comparison of the effects of* 0·1 *ml. i.v. of undiluted post- and pre-infection human ascitic fluid given on day* 3 *after giving Langat virus,* 500 *ICLD* 50 *i.p. or* 50 *ICLD* 50 *i.c., to* 4–6 *weeks old mice which had been given* 500 *rads of irradiation before infection*

Post-infection ascitic fluid				Pre-infection ascitic fluid			
Virus given i.c.		Virus given i.p.		Virus given i.c.		Virus given i.p.	
ADD*	Deaths	ADD	Deaths	ADD	Deaths	ADD	Deaths
9·3	23/23 (100)	17·6	3/9 (33)	8·3	23/23 (100)	13·2	9/9 (100)

Figures in parentheses indicate percentages.
* ADD = Average day of death.

received post-infection fluid six had no lesions and three had minimal lesions. Of the mice which had received pre-infection fluid one had no lesions, the remaining eight had lesions of grades 1 or 2. As in the previous experiments the lesions were atypical in that neuronal destruction was more prominent than is usual in association with such mild inflammatory changes. Thus, although there was the usual suppression of inflammation in the pre-infection fluid group, directly related to the irradiation, there was even less inflammation in the group which had received post-infection fluid after whole body irradiation and virus.

DISCUSSION

These experiments have shown that following irradiation antibody formation was delayed and partially suppressed. Irradiation also significantly increased the average survival time in those mice which had been given a small dose of virus i.c., and in those groups infected i.p. viraemia was prolonged and the brain virus titre continued to rise until death. However, the mode of death of the irradiated mice whether infected i.c. or i.p. was essentially non-paralytic. Therefore it seems reasonable to associate this lack of clinical neurological disturbance with the alteration in development of antibody. In addition the histological changes of encephalitis were also suppressed. Since the changes seen are largely due to the presence of mononuclear cells the destruction of these cells by irradiation must have some relevance to this.

Several workers have reported that irradiation may lead to increased susceptibility to infection with certain viruses (Imam & Hammon, 1957; Quilligan *et al.* 1963; Schneck & Berkovitch, 1965), and irradiation has been shown to inhibit antibody formation (Taliaferro, 1957) and interferon production (Julien & de Maeyer, 1966). The combined inhibition of antibody and interferon could well account for the prolongation of viraemia in the irradiated mice.

In our experiments, the viraemia in the control mice ended between the fifth and sixth day after infection and clinical neurological involvement began on the seventh day. Virus appeared in the brain on the fourth day, reached a peak on the fifth day and then gradually declined but was still present on the twelfth day. In contrast, viraemia in irradiated mice remained near its maximum from the second till the eighth day, and the brain virus titre rose steadily from the third day until death (Fig. 1). The higher brain titre of the irradiated mice may have been due to interferon suppression, but may have been apparent rather than real: in brains ground up after the appearance of antibody, a proportion of the virus would be neutralized before titration. Figure 1 shows that antibody, probably IgG, whether naturally produced or, as shown in the previous paper (Webb *et al.* 1968), artificially administered, plays a major part in the termination of the viraemia. Kundin (1966) and Tasker, Miesse & Berge (1962) have drawn similar conclusions with other arboviruses.

Langat virus in mice, in common with many other arboviruses, clearly has two distinct multiplication cycles: the first causing a high titre of virus in the blood, the second causing a high titre of virus in the brain. The lymph nodes draining the inoculation site area appear to be the site of primary virus multiplication (Malkova & Frankova, 1959 et seq.) and these and other lymph nodes are the likely sites for early antibody production (Litt, 1964, 1967). At the time of the main peak of viraemia antibody is circulating but as it will be rapidly combined with virus it is very difficult to detect. Evidence from other workers (Notkins, Mahar, Scheele & Goffman, 1966; Ashe & Notkins, 1966) suggests that considerable quantities of circulating virus may be, at this stage, combined with antibody but not neutralized. The virus-antibody complexes at this stage are small but as more antibody appears and combines they become larger and virus is neutralized. When antibody becomes detectable it is excess antibody, virus is then rapidly neutralized as it is released, and viraemia is no longer detectable. However, in irradiated mice antibody is diminished or delayed so that detectable viraemia is markedly prolonged. Depression of interferon production may have contributed both to the prolongation of viraemia and to the rather higher brain virus titres following irradiation.

The inflammatory changes in encephalitis are probably allergic reactions to virus-antibody complexes. These could be expected to occur where large complexes are deposited on the walls of small vessels, at points of serum filtration from the vessels and at places in the central nervous system (CNS) where virus released from cells encounters diffusing antibody. These are thus represented by aggregations of mononuclear cells in the meninges and perivascular spaces, together with proliferation of the glial elements at sites where there is primary neuronal damage from virus multiplication in the cells.

A small dose of specific antibody administered i.p. or i.c. before inoculation of virus was sufficient to protect the animals completely, even against large virus doses (Tables 2 and 7, Webb *et al.* 1968). In these circumstances the virus is presumably neutralized before it can disseminate and probably before it can even reach the regional lymph node. Thus, no detectable viraemia develops, the host does not fall ill, and there is no immune response. Administration of antibody after virus inoculation almost completely suppressed viraemia (Table 1, Webb *et al.* 1968). During the first 24 hr. the virus will have multiplied in the local lymph node and will have been spread via the blood to small numbers of cells throughout the body. Concomitant with this, antibody production will have begun in sensitized lymph nodes. Antibody administered at this stage will greatly slow the spread of virus from cell to cell by combining with a high proportion of released virus. Thus virus production and also the stimulation of antibody will be suppressed. Several types of antibody are formed during the course of a virus infection, some early and some late. The antibody formed later may well be the most important one because this antibody is at least partly antibody to the virus-antibody complexes formed earlier (Najjar, Robinson, Lawton, & Fidalgo, 1967) which are antigenically different from virus alone and which may contain an element of antibody to the host antibody itself. It may well be this antibody which combines together the antibody-coated virus particles and small complexes to produce complete neutralization of the virus and the larger complexes which we believe are responsible for the inflammatory aspect of the disease. Thus, under the experimental conditions, one would expect this late-type antibody and an accumulation of large virus-antibody complexes rather later in mice given antibody than in control mice. Also, because of the early general suppression of host antibody response, CNS virus multiplication can go ahead probably at a higher level for a longer time. The combination of these two factors could well cause the delay and the increase in incidence of paralysis and a greater intensity of lesions in certain areas. Both in animals given antiserum and in controls the meningitis and perivascular cuffing would be accounted for by a type of allergic reaction to virus-antibody complexes from the blood stream; similarly, glial reactions around areas of neuronal damage would be explained by an allergic reaction to complexes formed between diffusing antibody and released virus. The reaction may in fact be on a quantitative basis and the factor that determines which individual animal develops clinical encephalitis may depend on a critical numerical relationship between virus and antibody at a given time. We have shown that all inoculated mice have histological evidence of infection in the central nervous system. Virus was recovered from all but two of the brains; despite this, only a small proportion showed clinical signs of encephalitis.

We have not satisfactorily explained the relatively sudden non-paralytic death of the irradiated mice around the tenth day. However, their uniform death may have been due entirely to virus multiplication, uninhibited by either antibody or interferon, with consequent destruction of functional cells both in the CNS and other tissues. It is important to note that at the time of death of these animals both IgM and IgG were present and the possibility of some acute severe form of antigen-antibody reaction has not been excluded as a cause of death. If this is relevant, then it would appear that IgG is the more important antibody since the animals die within 48 hr. of the appearance of this.

The reason for the apparent reduction in paralysis rate on days 5 and 6 is not clear. It is known that the handling of mice produces stress which stimulates the release of steroids (Chang, 1965). This could well be advantageous to the mice at this stage by controlling oedema from the result of an antigen-antibody reaction. We have experiments in hand that tend to confirm that steroids given at this stage are beneficial. Also the addition of antiserum itself into the circulation at this time may alter circulating antigen-antibody complexes in a way beneficial to the mouse. This is also under investigation.

The site of inoculation of the antiserum may be relevant. The mice inoculated with antiserum i.p. had an increase both in frequency and intensity of lesions in the hind brain and spinal cord and in particular an increase in frequency and thickness of the perivascular cuffs in these areas. The serum had been inoculated into dermatomes supplied by the lumbar nerve roots. This situation appears to be analogous to that in poliomyelitis where trauma to a limb can lead to that part becoming the site of maximum paralysis.

The experiments in which animals were irradiated and then given antiserum may throw further light on the problem. Table 4 shows that antiserum, given i.p. on day 3 to mice which had been irradiated before inoculation with a small virus dose, gave considerable protection. Approximately 66% of these mice survived whilst none of the non-immune controls survived. As in the unirradiated mice which had been given post-infection ascitic fluid, clinical involvement when it did occur appeared late between the sixteenth and eighteenth days, compared to days 10–12 in the controls. This difference is reflected in the ADD shown in Table 4.

Kundin's experiments (1966) with an attenuated strain of Venezuelan equine encephalomyelitis virus indicate that there is a whole day's delay in the start of viraemia and an increase in virus titre following X-irradiation. As already stated there is evidence that the first phase of multiplication of many arboviruses may take place in the regional lymph node. If this is true for Langat virus in the mouse then the destruction of the lymphoid tissue by irradiation would deny the virus its first site for multiplication, lead to inefficient dissemination throughout the body and hence to delayed viraemia. Malkova (1962) using tick-borne encephalitis virus showed that this effect was most evident when a small virus inoculum was used. Similarly we found that, with a dose of 100 ICLD 50, viraemia was first detectable on the third day after inoculation. However, when viraemia was detectable on the day after infection (Fig. 1) the inoculum was 20,000 times larger than that given in Table 4. It therefore seems probable that the administration of antibody on either of the first 2 days, and perhaps also on the third day, might completely prevent the establishment of infection in at least a proportion of these mice. This is further supported by the total absence of any histological changes, including neuronal damage, in six of the nine mice examined on the twelfth day. However, when infection does become established in such mice the infection behaves as in unirradiated mice which have been given antiserum, with a late onset of paralysis.

In conclusion, we believe that the CNS is probably always involved to some extent in any generalized virus infection with an encephalitis virus (and perhaps with any virus as almost all have caused an occasional case of encephalitis) and that neuronal damage is caused both by virus multiplication in the neurones and by the inflammatory changes with their associated oedema and hypoxia. Except perhaps in the very highly susceptible (usually very young) animals, neuronal damage due to virus multiplication alone is probably seldom sufficient to cause severe paralysis, coma or death, although lesser signs and symptoms result. However, severe clinical illness and death may result when secondary neuronal damage due to inflammation is superimposed: the degree of recovery so common, for instance, in poliomyelitis when the patient survives the acute stage, is excellent evidence of how much of the dysfunction is attributable to inflammatory overlay. If acceptable means can be found to control the immunological responses and suppress the inflammatory aspect of these diseases, considerable amelioration of the clinical illness might result.

SUMMARY

1. Irradiation in a whole body dose of 200 rads or more increased the sensitivity of mice to intraperitoneal infection with Langat virus so that the LD 50 was increased to about the intracerebral LD 50.

2. In mice given 500 rads before infection: (*a*) viraemia was prolonged by about 5 days; (*b*) the IgM response was depressed; (*c*) the IgG response was delayed by about 3 days and depressed in titre; (*d*) virus concentration in the brain rose continuously until death on about the tenth day while in the controls it reached a peak on the fifth day then subsided; (*e*) histological changes in the CNS were delayed and minimal even at death; (*f*) irradiated mice died with little evidence of paralysis while the controls died with severe paralysis.

3. In irradiated mice, protection was observed when antibody was administered on the third day following infection. Antibody given on the 3 days after infection to control mice aggravated the disease.

4. The results in this and the preceding paper are discussed in relation to the pathogenesis of encephalitis. It is concluded that neuronal damage is caused both by virus multiplication in neurones and by damage superimposed by inflammatory changes with associated oedema and hypoxia. The inflammatory changes appear to be due to an allergic reaction to virus-antibody complexes formed in the circulation and in the central nervous system.

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A bacteriological assessment of meat, offal and other possible sources of human enteric infections in a Bantu township

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Two previous surveys undertaken at the South African Institute for Medical Research showed that many Bantu school children in rural and peri-urban areas (Bokkenheuser & Richardson, 1960; Richardson & Bokkenheuser, 1963) were infected with salmonellas and shigellas. It was suggested that the poor quality of water might possibly be incriminated in the spread of these organisms. In a third study subsequently carried out in an urban area using a similar group of school children, the only water available came from the Rand Water Board and was of excellent quality (Richardson, Koornhof & Hayden-Smith, 1966). The earning capacities of the families living in this area, although inadequate, were an improvement on the others. These factors, however, together with better hospital and clinic facilities, improved housing and sanitary conditions associated with urbanization, did not result in a noticeable decrease in the isolation of salmonella and shigella organisms.

Since human salmonellosis as a public health problem is attributable mainly to foods of animal origin, egg products, human carriers, food handlers and contaminated water (Sickenga, 1964; Bowmer, 1964; American Public Health Association, 1963) a study on the main items of food of animal origin consumed by the urban population was planned to assess their role in the high incidence of salmonellosis and shigellosis in the group of children investigated.

MATERIAL AND METHODS

Since previous surveys showed that the highest number of isolations of salmonellas and shigellas occurred during the warmer months, collections were arranged fortnightly for 14 months to see whether the isolated organisms followed a similar pattern.

Specimens

From the Johannesburg Municipal Abattoir

Each fortnight specimens of liver, spleen, lymph node, surface meat cut, bile and faeces were taken from a sheep and a bovine.

From the Offal Pool

Offal, consisting mainly of cattle and sheep tripe and intestine, plays an important part in the staple diet of the Bantu, both from an economic and nutritional point of view. As well as being cheaper, it is often eaten in preference to the conventional meat cuts. After slaughtering, the offal from the Municipal abattoir is rinsed and then transported to a building nearby known as the Offal Pool. From there, by various means of transport it is taken to the Soweto township near Johannesburg and sold to the inhabitants by licensed vendors.

From Soweto township

Soweto township is a housing scheme built by the government, the Johannesburg Municipality and other bodies, to accommodate a population of about 750,000 Bantu, many of whom are employed in various walks of life in Johannesburg. It is situated about 15–20 miles south-west of the centre of the city and essential services, including water-borne sewerage to all houses, are administered by the Johannesburg Municipality. The inhabitants are served by shops of all types to satisfy their daily needs.

Specimens of tripe, intestine and intestinal fat were collected fortnightly from two offal stalls and less frequently, specimens of liver, spleen and muscle. From the butcher shops an occasional specimen of polony, meat, mince and snoek (fish) was tested. In addition, specimens of sour milk, fowl and dog faeces were collected regularly for bacteriological assessment.

Bacteriological methods

About 2·0 g. of each animal tissue specimen was pulverized in Griffith's tubes using about 1·0 g. of sterile sea sand and then ground further after the addition of 1·0 ml. of sterile normal saline. All specimens including the sour milk, fowl and dog faeces were then tested for the following organisms.

Salmonellas and Shigellas

For the primary isolation, an S.S. agar and a Wilson and Blair plate were planted with a loopful from each suspension and incubated overnight. The ground-up tissue was introduced into selenite F medium and after incubation plated out on deoxycholate agar. From the S.S. and deoxycholate plate three non-lactose-fermenting colonies from each, if present, were tested for biochemical reactions. Likewise, characteristic salmonella colonies from the Wilson and Blair plates were tested after 24 and 48 hr. incubation. Those colonies conforming to salmonellas were typed serologically. Throughout the investigation no shigellas were encountered.

After the study had been in progress for 6 months it was noted that on no occasion were salmonellas found on the Wilson and Blair plate that were not present on either the primary S.S. or secondary deoxycholate plate. The use of the Wilson and Blair plate was then discontinued.

Coagulase-positive Staphylococcus aureus

From the original inoculum on Chapman's medium (1945) after 48 hr. incubation, typical colonies were picked off into plasma/mannite media. Positive cultures were then phage-typed.

Clostridium welchii

Willis & Hobbs medium (1959) was used for primary isolation. A secondary isolation using the same medium was made from Robertson's cooked meat medium the next day. All plates were incubated anaerobically. Colonies of *Cl. welchii* were confirmed by subculturing on a further Willis and Hobbs plate half of which contained *Cl. welchii* antitoxin; no opalescence caused by the lecithinase in this half indicated a positive result.

To test for heat-resistant *Cl. welchii* strains, the original ground suspension, at the end of all culturing procedures, was heated in a boiling water bath for 1 hr. Additional Willis and Hobbs plates were planted and examined the next day for the presence of *Cl. welchii* colonies. No typing of the strains was carried out.

Faecal Escherichia coli

All specimens submitted were tested for the presence of faecal *E. coli* by planting in MacConkey single-strength broth and, if positive with the production of acid and gas, were subcultured into brilliant green bile broth and peptone water. After incubation at 44° C. a positive indole reaction and gas production in the brilliant green bile broth was taken as evidence of faecal *E. coli*.

Bacillus cereus

Willis and Hobbs plates were inoculated from the original ground suspensions and incubated aerobically. The organisms were identified on colonial morphology with typical opalescence, caused by lecithinase production, and the characteristic microscopic appearance of the bacilli.

Antibiotic sensitivity

Using the disk method, all salmonella organisms isolated were tested for sensitivity to the following antibiotics: penicillin G (10 units), ampicillin (25 μg.), streptomycin (50 μg.), tetracycline hydrochloride (50 μg.), chloramphenicol (30 μg.), erythromycin (15 μg.) colistin (10 μg.), novobiocin (10 μg.), gentamycin (10 μg.). During the survey nitrofurantoin (100 μg.), kitasamycin (5 μg.), and naladixic acid (30 μg.) were added. A zone of inhibition of less than 2 mm. from the edge of the disk was taken to indicate a resistant organism. An Oxford strain of *Staphylococcus aureus* was used routinely as a control.

RESULTS

Of the specimens submitted from the thirty sheep (Table 1A) salmonellas were isolated from only two faeces. These were identified as *S. typhimurium* and *S. duval*. *Cl. welchii* isolations from tissue specimens ranged from nil in the lymph nodes to 5 (17 %) in the liver. In addition, this organism was recovered from 20 (67 %) faecal and 7 (23 %) bile specimens. *Bacillus cereus* was not evident in any of the tissue specimens and coagulase positive *Staph. aureus* very occasionally. Faecal *E. coli* were found extensively in most specimens.

The results of similar specimens from thirty cattle show that there were fewer isolations of all organisms, salmonellas excepted, than from the sheep (Table 1B). *S. carrau* was isolated from one lymph node and *S. london* from a specimen of faeces. The highest isolation from tissue specimens of faecal *E. coli* was 5 (17 %) from liver, and of *Cl. welchii* 3 (10 %), also from liver specimens. A heat-resistant strain of *Cl. welchii* was cultured from 1/12 (8 %) specimens of faeces.

Table 1. *Isolation of pathogens from sheep and cattle at abattoirs*

(Figures in parentheses indicate percentages.)

	Number of isolations of					
	No pathogens	*Salmonella* spp.	*Faecal E. coli*	*Cl. welchii**	*B. cereus*	Coagulase positive *Staph.*
			A. Specimens from thirty sheep			
Liver	6 (20)	0	24 (80)	5 (17)	0	2 (7)
Spleen	6 (20)	0	24 (80)	2 (7)	0	1 (3)
Lymph node	10 (33)	0	20 (67)	0	0	1 (3)
Surface meat cut	24 (80)	0	6 (20)	1 (3)	0	0
Bile	4 (13)	0	26 (87)	7 (23)	0	0
Faeces	0	2 (7)	29 (97)	20 (67)	6 (20)	0
			B. Specimens from thirty cattle			
Liver	23 (77)	0	5 (17)	3 (10)	0	0
Spleen	28 (93)	0	1 (3)	1 (3)	0	0
Lymph node	24 (80)	1 (3)	2 (7)	2 (7)	0	1 (3)
Surface meat cut	27 (90)	0	3 (10)	0	0	0
Bile	5 (17)	0	24 (80)	8 (27)	0	0
Faeces	0	1 (3)	30 (100)	29 (97)	9 (30)	0

* No heat-resistant *Clostridium welchii* isolated from sheep specimens. One heat-resistant strain o *Cl. welchii* from twelve specimens of cattle faeces tested.

Isolations from specimens from the Johannesburg Offal Pool and Soweto Township are presented in Table 2. From the Offal Pool, salmonellas were found in 14 (50 %) samples of tripe investigated and 11 (39 %) of intestine. Faecal *E. coli* were abundant and *Cl. welchii* were isolated from 11 (39 %) of tripe specimens and 22 (79 %) of intestine. No heat-resistant *Cl. welchii* were found and *B. cereus* and coagulase-positive *Staph. aureus* were cultured on only three occasions.

From the Soweto Offal stalls, similar results were obtained. Of note are the salmonella isolations, of which 26 (47 %) came from tripe samples, 13 (24 %) from intestine, 9 (16 %) from intestinal fat and 4 (20 %) from miscellaneous specimens. On 2 occasions out of 24 (8 %), heat-resistant *Cl. welchii* were found in intestinal fat. One specimen from an illegal unlicensed offal dealer yielded *S. typhimurium*, *B. cereus* and faecal *E. coli*.

Of twenty samples of polony, mince, meat cuts and snoek (fish) taken from butcher shops, no salmonellas were found and there were fewer isolations of the other organisms.

Specimens of sour milk were collected at random from street pedlars. No salmonellas were isolated, but 24 out of 28 samples (86 %) yielded faecal *E. coli*.

In the Bantu population, poultry is traditionally sold by the shopkeeper in its live state. The fowls are displayed in cages outside the shops and are fed by the shopkeeper until sold. Samples of fresh faeces were thus collected from the bottom of the cages for culture. Of 28 specimens, 4 (14 %) gave salmonellas, 22 (79 %) *Cl. welchii*, of which 3 out of 12 (25 %) were heat-resistant, and 7 (25 %) *B. cereus*. No coagulase positive *Staph. aureus* was isolated.

Table 2. *Isolation of pathogens from Johannesburg offal pool and from Soweto township*

(Figures in parentheses on each line indicate the total number of specimens examined.)

	Percentage of specimens showing					
	No pathogens	*Salmonella* spp.	Faecal *E. coli*	*Cl. welchii**	*B. cereus*	Coagulase-positive *Staph.*
Johannesburg offal pool						
Tripe (28)	0	50	96	39	0	4
Intestine (28)	0	39	100	79	4	4
Soweto offal stalls†						
Tripe (55)	0	47	98	74	13	7
Intestine (55)	0	24	100	96	13	2
Intestinal fat (57)	17	16	81	35	7	3
Liver, spleen and muscle (20)	0	20	100	75	5	0
Soweto butchers' shops						
Polony, meat, mince and snoek (20)	35	0	60	20	15	5
Other sources						
Sour milk (28)	14	0	86	4	7	0
Fowl faeces (28)	4	14	96	79	25	0
Dog faeces (28)	0	21	96	50	21	4

* Heat-resistant *Cl. welchii* were isolated from 2/24 specimens of intestinal fat, 3/12 specimens of fowl faeces and 7/12 specimens of dog faeces.

† One specimen from an unlicensed illegal offal vendor yielded *Salmonella typhimurium*, *Bacillus cereus* and faecal *Escherichia coli*.

Since dogs are known to be salmonella carriers, specimens of fresh faeces from some of the many township dogs were included in the survey. The samples came from the pavements, usually in the vicinity of the offal stalls which supplied the tripe and intestine specimens. Salmonellas were isolated from 6 (21 %) of 28 specimens, one of which had a double infection of *S. dakar* and *S. tel-el-kabir*, *Cl. welchii* from 14 (50 %), *B. cereus* from 6 (21 %) and there was one isolation of coagulase positive *Staph. aureus*. Heat-resistant *Cl. welchii* were found in 7 (58 %) of 12 specimens.

From all the specimens in the survey, ninety-three salmonellas were isolated comprising 28 different serotypes. Reference to Table 3 shows the frequency of isolations from various sources.

All salmonella strains were sensitive to tetracycline HCl, chloramphenicol, colistin, and gentamycin (Table 4). One strain of *S. duval* was resistant to ampicillin and streptomycin and another *S. duval* to ampicillin only. The conventional pattern was shown, all strains being resistant to penicillin G, novobiocin and kitasamycin, and 95 % to erythromycin.

Table 3. *Number, type and source of* Salmonella *strains isolated*

	Abattoir sheep and cattle specimens	Offal Pool tripe and intestine	Offal traders, all specimens	Dog faeces	Fowl faeces	Specimen from illegal offal trader	Total
S. typhimurium	1	9	9	.	1	1	21
S. london	1	1	13	.	1	.	16
S. newport	.	2	3	1	.	.	6
S. duval	1	1	3	.	.	.	5
S. montevideo	.	3	1	.	1	.	5
S. eastbourne	.	1	3	.	.	.	4
S. mission	.	1	2	.	.	.	3
S. stanley	.	2	1	.	.	.	3
S. labadi	.	1	2	.	.	.	3
S. saintpaul	.	.	3	.	.	.	3
S. tel-el-kabir	.	.	1	2	.	.	3
S. cerro	.	.	3	.	.	.	3
S. hato	.	.	2	.	.	.	2
S. poona	.	.	1	1	.	.	2
S. adelaide	.	1	.	.	.	.	1
S. johannesburg	.	1	.	.	.	.	1
S. anatum	.	1	.	.	.	.	1
S. pomona	.	1	.	.	.	.	1
S. paratyphi B	.	.	1	.	.	.	1
S. thompson	.	.	1	.	.	.	1
S. dublin	.	.	1	.	.	.	1
S. bulawayo	.	.	1	.	.	.	1
S. reading	.	.	1	.	.	.	1
S. minnesota	.	.	.	.	1	.	1
S. infantis	.	.	.	1	.	.	1
S. rowbarton	.	.	.	1	.	.	1
S. dakar	.	.	.	1	.	.	1
S. carrau	1	.	.	.	.	.	1
Total	4	25	52	7	4	1	93

Table 4. In vitro *resistance to antibiotics of salmonella strains isolated*

	No. of strains tested	Percentage resistant		No. of strains tested	Percentage resistant
Penicillin G	92	100	Colistin	92	0
Ampicillin	92	2	Novobiocin	92	100
Streptomycin	92	1	Gentamycin	92	0
Tetracycline HCl	92	0	Nitrofurantoin	14	0
Chloramphenicol	92	0	Naladixic acid	14	0
Erythromycin	92	95	Kitasamycin	67	100

DISCUSSION

Salmonellas

The prevalence of salmonellas in the various meat samples tested in this survey supplements the findings of various workers in other countries. (Report of a Working Party of the Public Health Laboratory Service, 1964; Kampelmacher, Guinée & Clarenburg, 1962; Galton, Steele & Newell, 1964.) Furthermore these workers have indicated the close correlation between animal and human infections.

Of the 240 specimens of cattle and sheep tissue from the abattoir examined over the period, only one specimen of bovine lymph node (0·4 %) yielded salmonellas (*S. carrau*). This compares favourably with the findings of the Working Party of the Public Health Laboratory Service in their investigation of meat samples from retail butcher shops.

The salmonella isolations from the tripe and intestines present an entirely different picture. From the Offal Pool half the specimens of tripe and 39 % of intestine yielded salmonellas. As one would expect, the number of salmonellas isolated from the distribution stalls in the township for those items was correspondingly high. Other meat samples sold from the stalls showed a 20 % salmonella incidence, probably due to contamination from the tripe and intestine through handling. No salmonellas were isolated from the twenty specimens from the butcher shops, which appeared to observe a higher hygienic standard.

Although the number of samples tested was small, the salmonella isolations are significant. The amount of tripe and intestine consumed annually by the population of Soweto is estimated at 12,000 tons, indicating the popularity of this food. Were this amount evenly distributed, consumption per head per diem would be 40 g., which would afford 8 g. animal protein (A. R. P. Walker, personal communication). In Britain, the corresponding figure for animal protein derived from *all* meat products is about 30 g. (Domestic Food Consumption and Expenditure, 1961). It will therefore be appreciated that the protein contributed to the Bantu by offal is highly significant. With a combined result of nearly 28 % salmonella isolations from all the specimens from the stall distributors and 44 % from the central pool, the risk to the population is obvious.

Previous investigations on the incidence of salmonellosis (Bokkenheuser & Greenberg, 1959) in various populations have shown that there are far more isolations of salmonellas in the hot summer months than in the winter. A similar trend was not observed in the recovery of salmonellas from these meat samples.

In keeping with reports of high isolations in other countries (Report of a Working Party of the Public Health Laboratory Service, 1964; Galton *et al.* 1964), *S. typhimurium* was the type most frequently found from the specimens of offal (18/77 = 23 %). This was followed by *S. london* (14/77 = 18 %) and *S. newport* (5/77 = 6 %) (Table 3).

In contrast, however, *S. dublin*, which is predominantly of bovine origin and relatively host specific, was only found in one instance. This organism has in the past been reported as being most common in cattle in South Africa (Henning, 1949), but has shown a decreasing trend in recent years (H. J. W. Botes, personal communication). An interesting feature of this survey was the single isolation of *S. johannesburg* in one of the Offal Pool specimens, since, during the period of the investigation, there were outbreaks of gastroenteritis due to *S. johannesburg*, particularly in infants and toddlers, in hospitals serving this area. During this period, a survey was conducted by Dr I. Spencer of the Johannesburg City Health Department and the South African Institute for Medical Research into the incidence of salmonellosis and shigellosis in children up to 2 years of age from clinics in Soweto. *S. johannesburg* was isolated on one occasion only (I. H. F. Spencer, personal communication). Unlike *S. typhimurium*, which is readily transmitted from animal to man, *S. johannesburg* appears to be far more host-specific to man.

The recovery of salmonellas, including *S. typhimurium*, from fowl faeces in this investigation serves to confirm another potential source of salmonellosis to the community. The salmonellas in fowl faeces might have been higher if media without bile salts had been used to allow all strains of *S. gallinarum-pullorum* to grow. It is well known that poultry, eggs and their subsequent products provide a large reservoir of salmonella organisms and adequate control is essential.

It is not surprising that, with the high salmonella incidence in the offal samples studied, there should be a relatively high recovery (21 %) from the dog faeces investigated. Butler & Herd (1965), investigating the incidence of human enteric pathogens in dogs in Alaska, found that approximately 16 % of family pets harboured salmonellas. Mackel *et al.* (1952) found that 15 % of 1626 household dogs were infected with salmonellas. Other workers (Watt & De Capito, 1950; Floyd, 1945) have found a much lower incidence.

Coagulase-positive Staphylococcus aureus

Phage typing carried out on thirteen of the sixteen isolations of coagulase-positive *Staph. aureus* isolated from all types of specimens studied showed that 6 (46 %) belonged to phage group III. According to Wilson & Miles (1964) coagulase-positive *Staph. aureus* organisms causing food poisoning may belong to this group and a smaller number to group IV, of which none were isolated in this survey. The significance of the small number isolated as a possible source of food poisoning is unknown.

Faecal Escherichia coli

Using the isolation of faecal *E. coli* as an indicator of faecal contamination, 62 % (74/120) of the liver, spleen, lymph node and meat-cut specimens from sheep at the abattoir were positive in contrast to the similar sampling from cattle which gave only 9 % (11/120). Since abattoir procedures on all animals are standard, one possible explanation may be the increased risk of tissue contamination with intestinal contents when working on smaller animals.

Nearly all specimens from the Offal Pool and Soweto Offal dealers showed gross contamination with faecal *E. coli*, as did 86 % of the specimens of sour milk tested.

Clostridium welchii

It is well known that *Cl. welchii* organisms cause food poisoning outbreaks. Vernon (1966) in reviewing food poisoning and salmonella infections in England estimated that half of the cases were from salmonella infections and a third were due to *Cl. welchii*. Hobbs & Wilson (1959), investigating the contamination of wholesale meat supplies, found heat-resistant *Cl. welchii* in 11 % boneless meat and 2 % carcass meat samples.

Theories as to the pathogenesis of the disease due to *Cl. welchii* are varied. Evidence exists for and against it being either intoxication or infection. Hobbs and her colleagues (1953), on examining many outbreaks of food poisoning, found that the organism responsible was a variant of Type A, being feebly toxigenic and heat-resistant. Nygren (1962) maintains that the food poisoning due to *Cl. welchii* is not caused by the organism or the exotoxin, but by phosphorylcholine produced by the enzymic action of lecithinase on lecithin in the food. If sufficient phosphorylcholine is present in the food, the intestine will be affected in 8–12 hr. by this substance, causing increased peristalsis and intestinal hurry. It is apparent, however, that more experimental studies are necessary to supplement Nygren's postulates (Nelson *et al.* 1966).

The extent of outbreaks in Soweto township of food poisoning due to *Cl. welchii* is unknown. Because of the high incidence of faecal *E. coli* in the samples from the Offal Pool and the offal vendors in the township, it is not surprising that the isolations of *Cl. welchii* are correspondingly high. Of the few samples in each group tested for heat-resistant *Cl. welchii*, only 2 of 24 (8 %) were isolated from intestinal fat. It is noteworthy that heat-resistant *Cl. welchii* were isolated from a quarter of the fowl faeces and over half of the dog faeces tested.

Bacillus cereus

Hauge (1950, 1955) and Christiansen (1951), as quoted by Wilson & Miles (1964), have produced evidence that foods containing large numbers of *B. cereus* may cause food poisoning. In all the specimens treated from all sources, fowl faeces yielded the highest number of isolations (25 %). However, in every specimen containing *B. cereus*, the number of organisms was so scanty that, in this survey, their significance is doubtful.

CONCLUSIONS

Although the number of specimens tested from the abattoir was small, the results obtained show that reasonable care is taken to ensure that the slaughtered animals are processed in a satisfactory manner. As far as the Offal Pool is concerned, it is clear that the tripe and intestines are not adequately treated after leaving the abattoir to render them comparatively free of faecal contamination.

The risk of infection, particularly salmonellosis, from this source is very real considering the vast quantities consumed and the lack of knowledge of modern hygienic habits. It is accepted that the transient human carriers among the Bantu population in Soweto township may constitute an important reservoir of salmonellas responsible for diarrhoea and can be as high as 14·5 % in school children in summer. (Richardson *et al.* 1966). Although salmonellas attack all age groups, infants and elderly people, whose resistance is weakened by other conditions, are more susceptible. In the Soweto township, the infant mortality rate (number of deaths of infants under one year of age per 1000 live births) was reduced over the last 10 years from 125·7 to 64·5, with a corresponding drop due to gastroenteritis over the same period from 41·4 to 19·3. The infant mortality rate in white children in Johannesburg due to gastroenteritis was 2·59 in 1957 and 1·02 in 1967 (I. H. F. Spencer, personal communication). Although the gastroenteritis infant mortality rate in the Bantu shows a marked improvement over the 10-year period it is high when compared with that of the white infants. The infected offal may well be directly responsible for this high incidence.

Health authorities should take cognizance of the findings of this study. It is realized that any scheme to improve the condition of the offal must be costly, but great care must be taken to ensure that the price to the consumer does not rise so high that they cannot afford this valuable source of protein.

SUMMARY

1. From the Municipal abattoir, specimens of liver, spleen, lymph node, surface meat, bile and faeces from a sheep and a bovine, were examined fortnightly for 14 months for the presence of possible pathogenic bacteria. The results suggest that slaughtering procedures are satisfactory.

2. Offal, consisting mainly of tripe and intestine, is eaten in large quantities by the Bantu population and is both nutritious and economical. The high incidence of salmonella isolations in the tripe (48 %) and intestines (29 %), and faecal *E. coli* and *Cl. welchii*, show that this commodity is distributed in an inadequately cleansed condition.

3. Faecal *E. coli* was isolated from 86 % of samples of sour milk collected from street pedlars.

4. Dog faeces collected from the township pavements yielded 21 % salmonellas, and faeces from fowls sold live by shopkeepers 14 %.

5. From the offal specimens, *S. typhimurium* (23 %) and *S. london* (18 %) were the salmonella types most frequently isolated. *S. dublin* was isolated on only one occasion.

6. Throughout the survey no shigellas were isolated.

7. The significance of the *Cl. welchii*, coagulase positive *Staphylococcus aureus* and *B. cereus* isolations from the various specimens tested is not known.

8. It is emphasized that although there is a definite need for improved treatment of the offal before distribution to the consumer, the resulting increase in cost must not be such as to deprive the population of this important source of protein.

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Elevated temperature incubation of enrichment media for the isolation of salmonellas from heavily contaminated materials

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INTRODUCTION

Alteration of incubation temperature is a well-known aid to purifying cultures of bacteria, although elevated temperature techniques have been mainly used to isolate thermophilic organisms (Wilson & Miles, 1964). A study of the literature will show, however, that some authors have increased the selectivity of their media for bacteria with an optimum growth temperature of 37° C. by raising the incubation temperature (MacConkey, 1908; Wilson, 1938). The procedure is not, therefore, a new one but is profitably revived from time to time. In 1953, Harvey & Thomson described a technique using selenite F broth incubated at 43° C. for the isolation of salmonellas. This paper mentioned three temperatures: 42, 43, and 44° C. For the conditions and media in use, it was suggested that 43° C. was the optimum for incubation with the proviso that 42° C. might be safer as 43° C. possibly represented the upper end of the useful temperature range.

We have now been using 43° C. incubation of selenite F broths for 14 years, sometimes alone and sometimes in conjunction with 37° C. We feel, therefore, that we can be objective about a technique which, in common with all isolation methods, has imperfections (Burman, 1967). For much of the material examined in our laboratory, the advantages outweigh the disadvantages. Other authors have recently found elevated incubation temperatures of value in enrichment and selective culture of salmonellas (Georgala & Boothroyd, 1964; Livingstone, 1965; Spino, 1966; Burman, 1967).

MATERIALS

The materials examined were:

(1) Gauze swabs (Moore, 1948) placed in Cardiff sewers 1953–54.

(2) Gauze swabs placed in drains from Cardiff and Barry abattoirs 1961–63.

(3) Samples of naturally polluted river water—the River Taff 1966–67.

METHODS

The gauze swabs were covered with nutrient broth and were pressed down with a sterile rod to extrude their absorbed fluid into the broth. This liquid was decanted and divided into two parts. Each part was diluted with an equal volume of double strength selenite F broth. One of the enrichment cultures was incubated at 37° C. and the other at 43° C. The samples were, therefore, paired. The river water samples were collected in 1 l. quantities and were brought to the laboratory within an hour. They were collected at the same sampling point each week at approximately the same time. At the laboratory they were divided into 10×100 ml. quantities and each 100 ml. was added to 100 ml. of double strength selenite F broth. Five of these enrichment cultures were incubated at 37° C. and five at 43° C. After exactly 24 hr. incubation the swab samples were subcultured to deoxycholate citrate agar, Wilson & Blair's bismuth sulphite agar and brilliant green MacConkey agar (Wilson & Blair, 1931; Harvey, 1956). The water samples, after 24 hr. incubation, were subcultured to S.S. agar (Difco or Oxoid) + 1% sucrose. The selective agars used for the swabs were incubated at 37° C and the brilliant green MacConkey plates were examined at 24 hr. The deoxycholate and Wilson & Blair plates were examined after 48 hr. incubation. The extra time was necessary for salmonella colonies to develop properly on freshly poured Wilson & Blair's agar and for optimum differentiation between proteus and salmonella colonies on deoxycholate citrate agar. The creamy brown colour sometimes shown by salmonella colonies on deoxycholate plates (Farrant, 1962) was found to be a useful distinguishing feature. The S.S. agars used for the river water were incubated at 40° C. for 24 hr. (Livingstone, 1965). A preliminary trial of this latter technique against Wilson and Blair's agar incubated at 37° C. had been promising and we wished to gain further experience of the method. Suspicious colonies were picked and examined in the usual way.

RESULTS

The results are given in Tables 1, 2 and 3. They are self explanatory. In Tables 1 and 2, the combined results of all three plating media are recorded for the two incubation temperatures of selenite F broth.

Table 1. *Sewage samples* 1953–54

Category	Number
Positive at 37° C.	31
Positive at 43° C.	54
Salmonellas isolated at 37° C. and not at 43° C.	1
Salmonellas isolated at 43° C. and not at 37° C.	24
Total samples	*163*

If we give no weight to results where procedures agree $\chi^2 = 21{\cdot}16$, $P = < 0{\cdot}0001$.

Table 2. *Abattoir samples* 1961–63

Category	Number
Positive at 37° C.	46
Positive at 43° C.	72
Salmonellas isolated at 37° C. but not at 43° C.	7
Salmonellas isolated at 43° C. but not at 37° C.	33
Total samples	501

If we give no weight to results where procedures agree $\chi^2 = 16{\cdot}9$, $P = < 0{\cdot}0001$.

Table 3. *River Taff water samples* 1966–67

	Temp. of incubation	
	37° C.	43° C.
Salmonellas isolated from	147	174
Salmonellas not isolated from	73	46
Total	220	220

These data cannot be arranged as in Tables 1 and 2. The specimens were derived from separate litre samples. Each litre was shaken vigorously and divided into ten equal parts, five of which were incubated at 37° C. and five at 43° C.

DISCUSSION

We have shown, for three different materials, that incubation of selenite F broth at 43° C. had advantages over incubation at 37° C. The specimens examined were all heavily contaminated with organisms other than salmonellas. A few faecal coli counts on the River Taff were over 18,000 per 100 ml. Each material was sampled over a period of at least 12 months. All seasons were, therefore, represented. The total observations covered 1953–67.

Lack of success with 43° C. incubation, in some laboratories, is probably explained on the basis of differences in sample or technique. Specimens that yield salmonellas easily at 37° C. are unlikely to yield more by the use of 43° C. Samples containing minimal numbers of salmonellas, or organisms in need of resuscitation, may be better examined at 37° C. (Burman, 1967). In the isolation of salmonellas from heavily contaminated materials, we constantly tread a technical tight-rope between sensitivity and selectivity. Others also have recognized this difficulty (Jameson, 1962).

Media preparation is also relevant to the use of 43° C. incubation. In our laboratory selenite F broth is sterilized by Seitz filtration (Hobbs & Allison, 1945). Heat is never used and the medium is crystal clear without a trace of red deposit. It is relatively non-toxic in that it usually allows multiplication and enrichment of *Shigella sonnei*. Other enrichment broths are, in our experience, more toxic than selenite F. If, for example, the formula of Rolfe's 'B' tetrathionate (Rolfe, 1946) is expressed in terms of molar tetrathionate (0·39 M) it will be noted that this medium is somewhat inhibitory (Knox, Gell & Pollock, 1943). A 'balanced' tetrathionate broth may easily be titrated to function very well at 43° C. and we have used such a medium at this temperature for the isolation of salmonellas from river water. Multiplication of media is, however, inconvenient for routine practice. The enrichment medium of Rappaport, Konforti & Navon (1956) can be adjusted to 43° C. incubation, but the results are not very satisfactory. We have not a great deal of experience with this medium, but in our hands it is toxic to *S. dublin*, *S. pullorum* and *S. typhi*. It therefore distorts the serotype pattern. This phenomenon is being currently investigated using selenite F broth as control.

If we can alter the formulae of enrichment media to function at 43° C., we can, just as easily, alter the incubation temperature to allow the use of more toxic enrichment broths. Spino (1966) for example, used a temperature of 41·5° C. with selenite brillant green broth and Kauffmann's tetrathionate broth. Adjustment of temperature may well be more convenient for laboratories using commercial media.

Plating media are also important in elevated temperature enrichment. The best results are obtained with brilliant green MacConkey agar. This medium suppresses the growth of *Proteus* species, does not react with selenite F broth with the production of an area of no growth at the site of heaviest inoculation and requires only 18–24 hr. incubation. It is particularly useful if multiple subculture from selenite F is used (Harvey & Phillips, 1955). Other authors have also found brilliant green agars useful (Georgala & Boothroyd, 1964; Spino, 1966). Deoxycholate citrate agar and S.S. agar are valuable if *S. dublin* is being searched for as some strains of this organism are inhibited by brilliant green MacConkey. Wilson and Blair's agar is not so well suited to 43° C. incubated selenite. This is, perhaps, because in Cardiff we do not ripen our plates in the refrigerator.

SUMMARY

In three separate series, samples were examined for salmonellas by culture in selenite F broths incubated at 37 and 43° C. The samples used were:

(1) Gauze swabs placed in sewage.
(2) Gauze swabs placed in drains in abattoirs.
(3) Sewage-polluted river water.

In each series the higher temperature gave better results.

The modification of tetrathionate broth for incubation at 43° C. and the adjustment of the incubation temperature to suit more inhibitory enrichment broths is discussed. The medium of Rappaport, Konforti & Navon (1956) is not suited to incubation at 43° C.

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Water-borne outbreak of viral gastroenteritis and Sonne dysentery

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Outbreaks of water-borne gastroenteritis and dysentery are relatively rare in Britain, yet many of the smaller water supplies in which water polluted with sewage is purified for domestic consumption are vulnerable to a breakdown in the purification process. An example of such a breakdown occurred at Montrose, a seaside town (population 10,800) situated 30 miles to the north-east of Dundee (population 185,000) in the County of Angus, Scotland.

THE OUTBREAK

History

On the evening of 18 August 1966 the general practitioners in Montrose received many calls to attend patients suffering from vomiting or diarrhoea or both these symptoms. Adults and children over 1 year of age were affected throughout the town and the cases were confined mainly to the town. A measure of the prevalence of the illness is seen in the fact that half of the night staff of a large canning factory failed to attend for work. On the following morning (19 August) one-quarter of the schoolchildren attending the three main schools were absent. The major wave of new calls to practitioners occurred on 19 and 20 August. There were no deaths in the outbreak. In one practice (population 4000) the number of new patients with diarrhoea or vomiting seen on the 6 days preceding the outbreak (13–18 August) were 0, 0, 3, 0, 1 and 2, and the numbers on the days 19–23 August were 49, 30, 3, 12 and 1.

It is estimated that 40–50 % of the population of Montrose was affected. In forty families visited routinely by health visitors on 22 August, 44 (27 adults and 17 children) out of 109 persons had been ill. In September, 553 food handlers were interviewed and 37 % gave a history of having had gastro-enteritis in the period 18–21 August and a further 8 % reported having been ill between 22 and 27 August.

Clinical features

The symptoms were colicky abdominal pain, nausea, vomiting, headache, general malaise and diarrhoea. Many patients had limb pains and a few were pyrexial. Two or three collapsed, with transient loss of consciousness. In those becoming ill during the first 24 hr. of the outbreak the predominant symptom was projectile vomiting, e.g. five or six episodes in as many hours, and one or two loose stools were passed during or just after the vomiting. In patients becoming ill later in the outbreak, diarrhoea was the principal feature and there was little or no vomiting. Epigastric tenderness was present in all patients seen by one practitioner. Blood and mucus were present in the faeces of only a few patients.

Treatment of most patients was with a sedative kaolin mixture, of which 32 gallons were sold in Montrose between 19 and 26 August. Many were also treated with tetracycline, but without obviously better results. Most patients showed full clinical recovery in 7–10 days and relapses were common.

Epidemiological investigations

In view of the simultaneous onset of the illness in large numbers of unrelated persons in the town, milk, food-stuffs and water were considered as possible vehicles of infection. The investigations of the early cases did not incriminate any of the three milk suppliers or any particular foodstuff, since all the suppliers of milk and foodstuffs distributed also to areas outside Montrose in which no cases occurred.

The widespread distribution of the cases led to the water supply being suspected as the vehicle of infection. Cases of the illness occurred throughout Montrose and in certain premises outside Montrose which were supplied with water from the Montrose main. Gastroenteritis did not occur, except in a few individuals who had visited Montrose, among the inhabitants of the nearby villages of Hillside and Ferryden, which depend on Montrose for food and milk but have a different water supply. No cases occurred in Sunnyside Royal Hospital, 2 miles from Montrose, which receives water drawn from the same supply as Montrose but chlorinated in a separate plant.

The Montrose water supply is filtered and chlorinated. The water is drawn from the River North Esk at Kinnaber and pumped to the water works near Sunnyside Royal Hospital. This river is polluted with sewage effluents from several villages along its course, Edzell Aerodrome and Stracathro Hospital. The water is treated with aluminium sulphate to precipitate organic matter and the pH adjusted with sodium carbonate. It is then subjected to mechanical pressure filtration through a battery of Bell's filters, chlorinated, normally to 0·4 parts per million, and held in a tank for 3–10 hr. before being distributed. Bacteriological results in the months preceding August had shown this treatment was adequate.

On 19 August, within 24 hr. of the start of the outbreak, the amount of free chlorine in a sample taken from a tap in the Burgh office was less than 0·1 p.p.m. and instructions were issued for the dose of chlorine to be increased to 1 p.p.m.

Samples of water taken at 2 p.m. and 2.30 p.m. on the same day from taps at two other points in the town were graded unsatisfactory on bacteriological examination; their confirmed *Escherichia coli* counts were > 180 and 90 per 100 ml. water. Samples taken on the following days were satisfactory, reflecting the effect of the increased dosage of chlorine.

The chlorination plant was examined some days later and a defect was found in the automatic switching gear which changes the supply of chlorine from an emptied cylinder to a standby full one. This defect allowed some chlorine to pass from the full to the empty cylinder, so that the whole of the calculated dose of chlorine did not pass into the water. No information could be obtained to show exactly when the breakdown took place but the unsatisfactory bacteriological findings on the water samples collected on 19 August demonstrated that the purification process had been inadequate for a period before the time of testing. In addition to the breakdown of chlorination, spates had occurred on 6, 10 and 14 August and the flood waters may have contributed to the failure of the process.

It was necessary to exclude, as a cause of the gastroenteritis, contamination of the water supply with pesticides and herbicides which had been sprayed on the agricultural land on the banks of the River North Esk just before the outbreak. Samples of canned vegetables known to contain water distributed on 16–19 August were tested in the laboratory of the Government Chemist for contamination with pesticides and herbicides but the tests were negative.

Bacteriological and virological investigations

Specimens of faeces were cultured on deoxycholate citrate agar plates and in tetrathionate broth and strains of *Shigella sonnei* were identified by standard biochemical and serological techniques. Antibiotic sensitivity tests were done with 'high level' Oxoid sensitivity disks and strains were considered to be sensitive if there was a zone of inhibition that extended more than 2 mm. beyond the edge of the disk. Colicine typing of strains of *Sh. sonnei* was kindly done by Dr R. R. Gillies, Bacteriology Department, University of Edinburgh.

For isolation of viruses the specimens of faeces were treated with penicillin and streptomycin and inoculated into secondary monkey kidney tissue cultures and primary human thyroid tissue culture. The thyroid cultures were prepared and maintained as described by Duncan (1960, 1961). The methods of isolation and identification of the viruses were as described by Weir, Jamieson & Green (1964). Coxsackie B antisera were from Burroughs Wellcome and Co., and the echovirus type 30 antiserum was kindly supplied by Prof. N. R. Grist and Dr Eleanor J. Bell, Regional Virus Laboratory, Ruchill Hospital, Glasgow.

RESULTS

Bacteriological results

Between 19 August and 19 September specimens of faeces were collected from 201 patients in Montrose who had vomiting or diarrhoea, 25 healthy convalescents who had a history of symptoms at the time of the outbreak, and 36 healthy contacts of patients. *Sh. sonnei* was isolated from 98 of the 201 patients (49%), 4 of the 25 convalescents (16%) and 8 of the 36 contacts (22%). Antibiotic sensitivity tests and colicine typing were done on 56 of the strains, and all 56 showed the same antibiotic sensitivity pattern and colicine type (type 4). The organisms were sensitive to chloramphenicol, nitrofurantoin and tetracycline, and resistant to ampicillin, kanamycin, neomycin, paromomycin, streptomycin and sulphonamide. The only variations were seen in 10 strains which showed borderline zones of inhibition with ampicillin disks, and one strain which was resistant to tetracycline. This antibiotic sensitivity pattern had not been seen previously in any of the 216 strains of *Sh. sonnei* tested from amongst those isolated in Montrose, Dundee or elsewhere in the County of Angus in the 2 years preceding the outbreak. Out of 195 strains isolated in Angus before the outbreak and colicine-typed by Dr R. R. Gillies only 6, isolated from patients in Montrose (1 patient 1964), Brechin (2 patients, 1964, 1964), and Dundee (3 patients, 1963, 1963, 1965), were colicine type 4. These 6 strains had a different antibiotic sensitivity pattern from that of the Montrose strain, being sensitive to ampicillin, kanamycin, neomycin and paromomycin, to which the Montrose strains were resistant. Moreover, these 6 strains did not produce the colicine active only against Abbott and Shannon's indicator strain 17 (DRL 10033) that is characteristic of the Montrose strains (personal communication from Mrs Janette Whyte.)

Thus the strains from the Montrose outbreak formed a homogeneous group, which differed in several characters from all strains previously found in the region. The findings are consistent with the view that the Montrose strains originated from a common source and that the parent strain was a recent importation from another region and did not originate by variation from a resident strain. By courtesy of Dr R. R. Gillies we obtained a collection of *Sh. sonnei* strains of colicine type 4 that had been isolated in Glasgow and Leeds in 1965 and 1966. Some of the Leeds strains had the same antibiotic sensitivity pattern and colicine activity as the Montrose strains, so that at least one source is known from which the Montrose strain could have been derived. Moore swabs (Moore, 1948) put in the river at the inlet of the Montrose water supply on several occasions after the outbreak did not yield *Sh. sonnei*.

In the period 13–29 September, i.e. 3–5 weeks after the outbreak, specimens of faeces were examined from 504 healthy individuals who lived or worked in Montrose and whose occupation involved the handling of food. Only three strains of *Sh. sonnei* were isolated and these strains belonged to colicine type 4 and showed the same pattern of antibiotic sensitivities as the strains from patients in the outbreak. The three carriers had presumably been infected at the time of the outbreak. Apart from this finding no further isolations of *Sh. sonnei* were made in Montrose. In April 1967, thirty specimens of faeces from patients with symptoms of gastroenteritis were examined and neither *Sh. sonnei* nor viruses were isolated from any of them. At the same time, four Moore swabs taken from the main Montrose sewers were negative for *Sh. sonnei*. The strain of *Sh. sonnei* that caused the outbreak apparently failed to establish itself in chronic carriers and disappeared from the town within a few months.

The 'Montrose' strain of *Sh. sonnei* did, however, spread from Montrose to other towns in Angus, namely Edzell, Brechin and Dundee, and it established itself for a period of at least several months as an endemic strain in Dundee, a town having a population eighteen times greater than that of Montrose. Out of 139 strains of *Sh. sonnei* isolated from patients in Dundee in the 6 months October 1966–March 1967, twenty-two were colicine type 4 and had the Montrose pattern of antibiotic sensitivities. Nine of the Montrose-type strains were isolated in a day nursery for pre-school children, which experienced a small outbreak of dysentery due to this organism. Throughout the whole period, however, the predominant strain of *Sh. sonnei* in Dundee was a colicine-untypable organism that did not show the Montrose pattern of antibiotic sensitivities. This strain has been the main *Sh. sonnei* strain in Dundee for at least 3 years.

In March 1967, a patient in Dundee yielded a strain of *Sh. sonnei* with the Montrose pattern of antibiotic sensitivities which unexpectedly was found to be colicine-untypable, and in the period March–May 1967 a further forty-five strains with the same characters were obtained in Dundee. Also in this period, seven strains of another new variety of *Sh. sonnei* were isolated from patients in Dundee; these were of colicine type 4 but they differed from the Montrose type-4 strain in being sensitive to kanamycin, neomycin and paromomycin. Type-4 strains with the Montrose pattern of antibiotic sensitivities have not been isolated since March 1967, and the organism seems now to have disappeared from Dundee. The two new varieties of *Sh. sonnei* just mentioned may be importations into the region, but it also seems possible that they have been derived by variation from the Montrose organism, the one by a loss of colicine production, the other by a loss of resistance to kanamycin, neomycin and paromomycin.

Virological results

Specimens for virus isolation were obtained from 32 patients in Montrose who first became ill between 10 and 29 August; 3 of these patients became ill before the outbreak (10, 16, 17 August); 18 were involved in the main outbreak (19–21 August) and 11 in the following week.

Sixteen (50 %) of the patients were found to be excreting viruses and 7 (21 %) to be excreting *Sh. sonnei* (Table 1). Eighteen of the patients were under 20 years of age and the 7 isolations of *Sh. sonnei* were obtained in this group together with 7 of the virus isolations.

The first case was a woman who became ill on 10 August with a febrile illness with diarrhoea, 8 days before the outbreak began. A specimen of faeces taken 3 weeks later at a time when the diarrhoea was still continuing yielded Coxsackie virus type B5. The cases taking ill with a feverish illness on 16 and 17 August were twins aged 21 months; they developed diarrhoea on the following day. The faeces of both contained Coxsackie virus type B6 when they were examined three weeks later. These three cases occurred before 18 August and indicate that Coxsackie viruses types B5 and B6 were already in the community when the outbreak took place.

Faeces from eighteen cases who were taken ill between 18 and 21 August were examined for viruses and the specimens yielded four echoviruses type 30, four Coxsackie viruses type B6 and three strains of *Sh. sonnei*. Specimens from eleven patients becoming ill on 24–29 August yielded one strain each of Coxsackie viruses type B5 and B6, three strains of echovirus type 30, and four of *Sh. sonnei*. Two patients yielded both *Sh. sonnei* and echovirus type 30 from the same specimen of faeces.

Table 1. *Date of onset of illness and isolations of viruses and* Shigella sonnei *from thirty-two patients whose faeces were examined for both bacteria and viruses*

	Number of patients yielding stated pathogen from faeces, who first became ill on the following days of August											
Pathogen isolated	10	16	17	18	19	20	21	24	25	26	27	29
Echovirus type 30	0	0	0	1	0	1	2	0	0	0	2	1
Coxsackie virus B5	1	0	0	0	0	0	0	0	1	0	0	0
Coxsackie virus B6	0	1	1	0	1	1	2	0	0	0	1	0
Sh. sonnei	0	0	0	3	0	0	0	1	0	0	2	1
None of above	0	0	0	3	0	2	2	1	0	1	0	0

Two patients on 27 August had both echovirus type 30 and *Sh. sonnei* in their faeces.
The first day of the outbreak was 18 August.

Table 2. *Principal symptoms correlated with isolation of pathogens in thirty-two patients from whom faecal specimens were submitted for examination*

Principal symptoms	Number of cases	Number of isolations of a pathogenic organism
Gastroenteritis	14	4 *Sh. sonnei*
Diarrhoea	10	5 Coxsackie virus B6 1 Coxsackie virus B5 2 Echovirus type 30 1 *Sh. sonnei*
General malaise and enteritis	4	2 Echovirus type 30 1 Coxsackie virus B5 2 Coxsackie virus B6
Blood and mucus	2	2 Echovirus type 30 } double 2 *Sh. sonnei* } infections
Headache and dizziness	2	1 Echovirus type 30

Table 2 shows the relation between the organisms isolated from the thirty-two cases and the clinical diagnoses. The strains of *Sh. sonnei* were found in the patients with gastroenteritis and those having blood and mucus in the stools. Most of the Coxsackie viruses were associated with diarrhoeal illnesses. The echovirus type 30 strains were associated with enteritis illnesses except for one isolated from a boy with dizziness and fever, suggesting a meningitic type of illness.

Attempts to isolate viruses from Moore swabs taken from the river near the water supply inlet were not successful.

DISCUSSION

This explosive outbreak of gastroenteritis that within two days probably affected about 4000 persons in a town of 10,800 inhabitants was almost certainly due to water-borne infection. The reasons for this conclusion are: (1) the agreement between the distribution of cases and that of the main water supply to the town, (2) the absence of a relationship between the distribution of cases and the distribution of food and milk supplies, (3) the demonstration of a fault in the equipment used to chlorinate the water supply, (4) the demonstration that the water reaching the town at the time of the outbreak contained an inadequate amount of residual chlorine, and (5) the demonstration that the water reaching the town at the time of the outbreak contained large numbers of viable *Escherichia coli* organisms and, therefore, that there had been a failure in its purification.

The nature of the water-borne pathogens is not fully known but *Shigella sonnei* was almost certainly the cause of about half of the cases since it was isolated from the faeces of 49 % of patients subjected to bacteriological examination. Possibly *Sh. sonnei* caused the illness in a further proportion of cases, but was not isolated in these cases because of the technical limitation inherent in the single bacterial culture attempted. The outbreak could be attributed wholly to *Sh. sonnei* if it were assumed that the proportion of missed cases was 51 %, but we consider it unlikely that the efficiency of our cultural methods was as low as that.

On the other hand, our isolation of viruses from half the faeces examined virologically suggests that a proportion of the illnesses in the outbreak may have been caused by water-borne viruses. Since the purification process was defective and the water supply known to be heavily polluted with *Esch. coli* organisms, it is very probable that enteroviruses were also present. Although two Coxsackie B viruses were present in the community before 18 August, the echovirus type 30 infections appear to have started along with the *Sh. sonnei* infections on the 18th August and echovirus type 30 accounted for seven out of the 16 virus isolations.

Outbreaks of water-borne *Sh. sonnei* have not occurred often in Great Britain. Green & Macleod (1943) described an explosive epidemic among 400 persons in a town of 10,000 inhabitants. *Sh. sonnei* was isolated from some patients and also from a laboratory tap. The tap sample was satisfactory in other respects by usual bacteriological tests. The cause of the epidemic was inadequate chlorination of deep well water. The symptoms were similar to those in Montrose, i.e. prostration, sickness and vomiting, colic and diarrhoea.

Ross & Gillespie (1952) reported an outbreak of water-borne gastroenteritis affecting employees in a factory; three specimens of stool were examined from sixty-six patients, *Sh. sonnei* being isolated from nine patients, *Salmonella typhimurium* from one patient and *Shigella flexneri* from one patient. *Sh. sonnei* was later isolated from river water. The majority of cases were thought to be due to 'non-specific' causes as no other pathogens were demonstrated. The cause of the outbreak was the contamination of the town water mains by crude river water through a cross-connexion in the factory between two pipes, one carrying river water at 240 lb. pressure, the other carrying town water at 25–30 lb. pressure.

Murchison (1966) described an outbreak of *Sh. sonnei* dysentery in a village community in Scotland in which 25–30 % of people developed dysentery. Water from a well contaminated by flood-borne excreta played a part in the spread of the disease.

In the United States, Freitag (1960) reported a sudden and extensive outbreak of dysentery due to *Sh. sonnei* in Ravena in Albany County; 45–50 % of the population had a clinical illness, there being about 1400 cases in a population of 3200 inhabitants. The outbreak was due to heavy pollution of the water supply with *Sh. sonnei* and a breakdown in the treatment of the water, a period of time from a few hours to a day elapsing during which no chlorine and possibly no alum was added to the raw water.

An outbreak of Sonne dysentery in a town in the Ukraine was reported by Sorvina in 1946; sewage contamination of a piped water supply was probably the cause but *Sh. sonnei* was not demonstrated in the water. Dordević, Sokolovski & Miladinović (1965) described four water-borne epidemics of dysentery that took place in 1962–64 in one unit of a military garrison. The morbidity rates varied from 126·4 to 312·4 per 1000 population. One outbreak was due to *Sh. sonnei* the others to *Sh. flexneri* type Z or *Sh. schmitzii*.

There is evidence that enterovirus infection may be water-borne. Lamb, Chin & Scarce (1964) tested 164 weekly samples of sewage and river water collected from July to November 1962. Eighty-one (49 %) of samples were found to contain viruses. Raw sewage yielded the highest frequency of positive samples (80 %); 52 % of effluent samples were positive. About one-quarter of the river water samples contained viruses, Group-B Coxsackie viruses and polio-viruses being most commonly found. Enteroviruses can survive in river water for at least 1 mile from the outfall. Since enteroviruses apparently can survive in water and in sewage, it is obvious that they can be distributed by an inadequately chlorinated water supply and it is possible that the Coxsackie virus B6 and echovirus type 30 isolated in Montrose had been distributed in this way.

The conditions of filtration and chlorination required to render water free from infective virus are not precisely known. There is evidence that the enteroviruses require a higher dose of chlorine and a longer holding time than is required to kill the bacteria observed as a guide to cleanliness of water. Kelly & Sanderson (1958) showed that complete elimination of enteroviruses was not achieved by the usual conditions for bacterial disinfection of water supplies, i.e. free residual chlorine concentration of 0·2 p.p.m. for 10 min. contact at pH 7. A longer period of contact was necessary. Isherwood (1965) has shown that the inactivation of Coxsackie virus type A13 required a combination of activated sludge treatment using a very high sludge volume index in excess of 700 ml./l. followed by sand filtration and chlorination to a residual level of 0·5 p.p.m. with a contact time of 8 hr. McLean & Brown (1966) have shown that currently used methods of filtration do not remove viruses and that high-level chlorination is required; with about 0·8 p.p.m. residual chlorine it was found that small amounts of polio-virus type 1 were rapidly inactivated in running bath water.

The ability of the types of viruses isolated in Montrose to cause an illness characterized by acute vomiting and diarrhoea is not fully established. However, in a localized outbreak in Italy, Felici *et al.* (1962) recovered Coxsackie virus B3 from 33 % of infants and children with summer diarrhoea and from 12 % of healthy contacts. A break in the sewage system appeared to be the primary cause in this outbreak. Presumably the Coxsackie B5 and B6 viruses isolated in Montrose were capable of giving the same type of clinical illness. It has not been suggested previously that water-borne echovirus type 30 might give rise to an intestinal type of illness. Irvine, Irvine & Gardner (1967) have described an outbreak in a general practice in which echovirus type 30 was isolated from a majority of cases in which the principal symptoms included headache, malaise, muscle pain, vomiting and abdominal pain. These symptoms are more of a 'gastro-intestinal' type than those usually associated with this virus and are similar to the symptoms found in some of the cases in Montrose.

In this outbreak the infecting agents were *Sh. sonnei* and three enteroviruses. None of the agents isolated produced life-endangering illnesses, but where *Sh. sonnei* and these three enteroviruses can go, so can *Salmonella typhi*, poliovirus type 1, infectious hepatitis virus, *Entamoeba histolytica* and other agents producing severe disease. It is clear, therefore, that more thought and attention must be given to the purification of water supplies drawn from sources liable to pollution with sewage. The points of importance would appear to be adequate filtration, adequate chlorination and adequate holding time after chlorination. Supervision of the purification process should be adequate to guard against the possibility of accidental breakdowns, and this may be difficult to achieve in small water-supply undertakings. The observations in the literature indicate that a higher residual chlorine level than is used for bacteriological disinfection is required to render viruses inactive. If mechanical filters are used in place of the more efficient biological (slow sand) filters, it is particularly important that high levels of chlorine should be used.

SUMMARY

1. An explosive outbreak of gastroenteritis that affected 40–50 % of people in a town of 10,800 inhabitants (Montrose) is described.

2. There is epidemiological evidence that the outbreak was water-borne. The chlorination of the water supply was faulty at the time of the outbreak.

3. Echovirus type 30 and two types of Coxsackie B viruses were isolated from sixteen out of thirty-two patients examined.

4. *Shigella sonnei* was isolated from the faeces of 110 out of 262 patients and contacts examined. Fifty-six strains tested for colicine activity were all colicine type 4 and had the same antibiotic sensitivity pattern.

5. This particular strain of *Shigella sonnei* (Montrose strain) spread to surrounding areas, although it disappeared quickly from Montrose.

6. The problem of inactivating virus in water supplies contaminated with sewage is discussed briefly.

We wish to acknowledge the help given by the general practitioners of Montrose and the staff of the Public Health Department of the County of Angus in the collection of specimens and information during the outbreak. We would also like to thank the Laboratory staffs of the diagnostic laboratory and virus laboratory of the Bacteriology Department, University of Dundee and the staff of the laboratory, Stracathro Hospital, for invaluable assistance. We are indebted to Dr R. R. Gillies, Epidemiology Unit, Bacteriology Department, University of Edinburgh, who kindly carried out colicine typing for us and supplied us with strains from his collection, and to Dr R. M. Gordon, Senior Medical Officer, Scottish Home and Health Department and Mr H. F. Scrimgeour, M.I.C.E., A.I.W.E., Engineering Inspector Scottish Development Department, for their assistance during the investigation.

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Survival of wound pathogens under different environmental conditions

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Moisture is known to favour the survival of Gram-negative bacilli in the environment, while dry conditions favour the survival of Gram-positive cocci (Hellat, 1948; Bardsley, 1948; Lidwell & Lowbury, 1950). Lowbury & Fox (1953) found that the proportion of bacteria which died when their suspending medium evaporated to dryness was much greater with a suspension of *Pseudomonas aeruginosa* than with one of *Staphylococcus aureus* or *Streptococcus pyogenes*; the death-rate of the Gram-negative bacilli which had survived drying and which were exposed for a further period under atmospheric conditions was, however, no greater than that of the survivors in dried suspensions of streptococci or staphylococci.

Among the questions left unanswered in the earlier studies were (1) the consistency, on replicate testing, of differences in survival of different strains of *Ps. aeruginosa*, (2) the relative capacity of bacterial cells, which have survived one exposure to drying, to survive a second drying, and (3) the relative capacities of *Ps. aeruginosa* and of *Staph. aureus* to survive in identical moist environments. These questions are considered in this paper. The relevance of the survival of *Ps. aeruginosa* and *Staph. aureus* under different environmental conditions is also discussed.

SURVIVAL OF ORGANISMS ON ATMOSPHERIC DRYING

Methods and materials

Bacterial strains

The Gram-positive cocci tested were three strains of *Staph. aureus* (phage types 52A/80, 3C and 80/81) and two strains of coagulase-negative staphylococci (micrococci). The Gram-negative bacilli included four strains of *Ps. aeruginosa* isolated from burns (phage types 21/68/F7, 119x, 21/68/col.21 and an untypable strain), two strains from wounds (phage types 73/119x/+ and 68/73/119x), two from urine (phage type 7/73/M4 and type unknown), one from faeces (phage type 44/68/109/119x/1214), and six strains isolated from the environment (three from dust in a burns ward, phage types F8, 21/68 and type unknown, and three from ward floor cultures, phage types 7/31/352, 68/73/109/119x/+ and untypable). Two strains of *Escherichia coli* (FE1 and FE2) and a strain of *Klebsiella aerogenes* (FKa) were also studied.

Preparation of suspensions

Cultures of the bacteria in 3 ml. infusion broth incubated at 37° C. for 24 hr. were centrifuged at 2000 rev./min. for 30 min., washed with deionized water and resuspended in deionized water to give a viable count of approximately 2×10^7 organisms/ml.

Exposure to drying

Sterile, grease-free coverslips (16 mm. diameter) were mounted in pairs on glass slides. Approximately 0·03 ml. amounts of the freshly prepared bacterial suspensions were dropped on the coverslips from standard dropping pipettes, and the drops were spread over the surface of the coverslips with a sterile platinum-wire loop. The coverslips, on their slides, were then placed under the lid of a cardboard box and left to dry at room temperature (approximately 25° C.); relative humidity varied from 35 % to 50 %.

With each strain in the first experiment six coverslips were extracted immediately, six after drying for 2 hr. and six at 24 hr. In the second experiment two coverslips were extracted immediately and two at 15 min. or 30 min. intervals for the duration of 4 hr. In an experiment to show long-term survival, one of a set of prepared coverslips was sampled each week over a period of 8 weeks.

Extraction of coverslips and bacterial counts

Coverslips carrying the bacterial suspensions were transferred to 10 ml. quantities of broth in screwcap bottles (Universal containers). After the lids had been screwed on firmly, the bottles were shaken by hand for 1 min.; 1 in 10, 1 in 100 and 1 in 1000 dilutions of the extracts were made in Ringer's solution, and three 0·03 ml. amounts of each dilution and of the undiluted extract were dropped from standard dropping pipettes on nutrient agar or horse-blood agar plates. These were incubated at 37° C. for 24 hr. and the numbers of colonies from each drop were counted. With the suspensions of *Ps. aeruginosa* that had been left after drying for several weeks, the coverslips were transferred to 10 ml. quantities of broth and shaken for 1 min. and the suspensions were incubated for 24 hr. or longer before loopfuls were spread on nutrient agar plates. The plates were incubated for 24 hr. and then, where necessary, growth of *Ps. aeruginosa* was confirmed by growth and fluorescence on subculture to a selective medium (Brown & Lowbury, 1965). Viable counts were not made, since the numbers of survivors were very small; presence or absence of growth only was recorded.

Results

Survival of organisms in films exposed on coverslips for 2 hr. and for 24 hr.

The percentage survival on exposure to atmospheric drying of suspensions of *Staph. aureus*, coagulase negative staphylococci and *E. coli* is shown in Table 1. The survival of the Gram-positive cocci was found to be variable, but all showed a higher percentage survival at 2 and 24 hr. than the strain of *E. coli*, or the strains of *Ps. aeruginosa* shown in Table 2. One strain of coagulase negative

Table 1. *Survival of* Staphylococcus aureus, *micrococci and* Escherichia coli *on drying of suspensions*

Organism	Antibiotic resistance*	Phage type	Viable counts of bacteria on coverslips† 2 hr. (a)	2 hr. (b)	24 hr. (a)	24 hr. (b)	Survivors (%) 2 hr.	Survivors (%) 24 hr.
Staph. aureus FS 1	P.T	52A/80	$8{\cdot}9\times10^4$	$8{\cdot}6\times10^4$	$2{\cdot}0\times10^4$	$2{\cdot}5\times10^4$	31·5	7·70
Staph. aureus FS 2	Sens.	3c	$1{\cdot}5\times10^5$	$1{\cdot}6\times10^5$	$5{\cdot}7\times10^3$	$6{\cdot}1\times10^3$	24·7	1·08
Staph. aureus FS 3	T.N.Ne	80/81 (1000 R.T.D.)	$2{\cdot}9\times10^5$	$3{\cdot}0\times10^5$	$5{\cdot}7\times10^4$	$5{\cdot}9\times10^4$	100	34·5
Staph. aureus FS 3‡	T.N	80/81 (1000 R.T.D.)	$1{\cdot}8\times10^5$	$2{\cdot}2\times10^5$	$4{\cdot}1\times10^4$	$3{\cdot}9\times10^4$	100	38·0
Micrococcus (white)	.	.	$1{\cdot}4\times10^5$	$1{\cdot}1\times10^5$	$8{\cdot}1\times10^4$	$7{\cdot}9\times10^4$	53·4	32·3
Micrococcus (yellow)	.	.	$4{\cdot}9\times10^5$	$5{\cdot}0\times10^5$	$4{\cdot}5\times10^5$	$4{\cdot}1\times10^5$	99·0	87·0
E. coli FE 1	.	.	$2{\cdot}6\times10^4$	$2{\cdot}3\times10^4$	$1{\cdot}3\times10^3$	$1{\cdot}4\times10^3$	2·2	0·1

* P = Penicillin, T = tetracycline, N = novobiocin, Ne = Neomycin, Sens. = sensitive to all antibiotics tested.

† (*a*) and (*b*) show the mean results from coverslips 1, 2 and 3 and from coverslips 4, 5 and 6 respectively, with each strain tested; they are presented as simultaneous replicate experiments.

‡ A neomycin-sensitive variant isolated after storage of the culture that was resistant to T, N and Ne.

Table 2. *Survival of* Pseudomonas aeruginosa *from patients and from dry environment on drying of suspensions*

			Viable counts of bacteria on coverslips*					
			2 hr.		24 hr.		Survivors (%)	
Strain	Source	Phage type	(*a*)	(*b*)	(*a*)	(*b*)	2 hr.	24 hr.
F 1	Burn	21/68/F7	$0{\cdot}8 \times 10^4$	$1{\cdot}3 \times 10^4$	$1{\cdot}0 \times 10^2$	$1{\cdot}0 \times 10^2$	1·74	0·020
F 2	Burn	119x	$2{\cdot}4 \times 10^4$	$2{\cdot}2 \times 10^4$	$3{\cdot}9 \times 10^2$	$4{\cdot}3 \times 10^2$	5·30	0·097
F 3	Burn	Unknown	$6{\cdot}3 \times 10^4$	$6{\cdot}1 \times 10^4$	$5{\cdot}6 \times 10^2$	$5{\cdot}9 \times 10^2$	9·40	0·089
F 4	Burn	21/68/col. 21	$2{\cdot}7 \times 10^4$	$3{\cdot}1 \times 10^4$	$5{\cdot}9 \times 10$	$6{\cdot}6 \times 10$	3·00	0·008
F 5	Wound A	73/119x/+	$2{\cdot}2 \times 10^4$	$2{\cdot}3 \times 10^4$	$2{\cdot}1 \times 10^3$	$1{\cdot}8 \times 10^3$	3·06	0·251
F 6	Wound B	68/73/109/119x	$4{\cdot}0 \times 10^4$	$4{\cdot}3 \times 10^4$	$3{\cdot}6 \times 10^3$	$3{\cdot}0 \times 10^3$	3·90	0·300
F 7	Urine	7/73/M4	$1{\cdot}5 \times 10^4$	$2{\cdot}1 \times 10^4$	$2{\cdot}7 \times 10^3$	$2{\cdot}4 \times 10^3$	3·20	0·480
F 8	Urine	Unknown	$3{\cdot}4 \times 10^4$	$3{\cdot}1 \times 10^4$	$1{\cdot}3 \times 10^2$	$1{\cdot}5 \times 10^2$	15·00	0·060
F 9	Faeces	44/68/109/119x/1214	$1{\cdot}0 \times 10^4$	$1{\cdot}2 \times 10^4$	.	.	0·77	.
F 10	Dust (Burns Ward)	Unknown	$2{\cdot}7 \times 10^5$	$3{\cdot}0 \times 10^5$	$5{\cdot}1 \times 10^3$	$4{\cdot}5 \times 10^3$	19·80	0·315
F 11	Dust (Burns Ward)	F 8	$3{\cdot}8 \times 10^4$	$3{\cdot}9 \times 10^4$	$2{\cdot}8 \times 10^3$	$2{\cdot}9 \times 10^3$	10.20	0·760
F 12	Dust (Burns Ward)	21/68	$3{\cdot}3 \times 10^4$	$3{\cdot}8 \times 10^4$	$1{\cdot}6 \times 10^2$	$1{\cdot}8 \times 10^2$	5·80	0·016
F 13	Ward floor	7/31/352	$3{\cdot}2 \times 10^5$	$3{\cdot}0 \times 10^5$	$3{\cdot}2 \times 10^2$	$3{\cdot}3 \times 10^2$	26·80	0·110
F 14	Ward floor	Not typable	$6{\cdot}0 \times 10^4$	$6{\cdot}3 \times 10^4$	$1{\cdot}4 \times 10^2$	$2{\cdot}0 \times 10^2$	4·20	0·012
F 15	Ward floor	68/73/109/119x/+	$1{\cdot}9 \times 10^4$	$2{\cdot}1 \times 10^4$	.	.	5·80	.

* (*a*) and (*b*) show the mean results from coverslips 1, 2 and 3 and from coverslips 4, 5 and 6 respectively, with each strain tested; they *are presented as simultaneous replicate experiments.*

staphylococcus showed a particularly high survival rate at 24 hr. (87 %); in contrast one strain of *Staph. aureus* (FS 2) showed only 1·08 % survivors at 24 hr. A neomycin-sensitive variant of *Staph. aureus*, strain FS 3, showed a survival rate similar to that of the neomycin-resistant parent strain. Replicate coverslips showed similar survival patterns for the same strains.

Table 2 shows the survival of strains of *Ps. aeruginosa* isolated from patients and from the environment. The mean percentage survival at 2 hr. of strains isolated from patients ($5{\cdot}04 \pm 2{\cdot}22$) was lower than the mean percentage survival at 2 hr. of strains isolated from the environment ($12{\cdot}1 \pm 3{\cdot}98$) ($t = 3{\cdot}83$, $P < 0{\cdot}01$, $> 0{\cdot}001$). Strains F 5 and F 6, apparently of the same phage type but isolated from two different wounds (A and B), showed similar survival rates at 2 hr. Environmental strain F 15 was of the same phage type as strains F 5 and F 6 and showed approximately the same survival rate (5·8 %). Strains F 6 and F 15 were excluded from the analysis because their types duplicated those of other strains in the series.

The percentage survival of all strains on exposure for 24 hr. was very low (< 1 %).

Replicate tests of strains F 3, F 8 and F 13 made on successive days showed similar results (see Table 3).

Table 3. *Survival of three strains of* Pseudomonas aeruginosa *on drying: replicate tests on successive days*

			Survivors (%)			
			Test 1		Test 2	
		Phage type	2 hr.	24 hr.	2 hr	24 hr.
F 3	Burn	.	9·4	0·089	8·8	0·082
F 8	Urine	.	15·0	0·060	19·2	0·082
F 13	Ward floor	7/31/352	26·8	0·110	21·5	0·037

Survival of organisms at intervals during periods of drying (4 *hr.*)

Figure 1 shows that the number of survivors rapidly decreased during the period of drying; this occurred 30–90 min. from the start of the experiment, when the suspensions became visibly dry, after which there was little or no further death of bacteria. The percentage survival of *Staph. aureus* (phage type 52A/80) and of the strain of coagulase-negative staphylococcus tested was much higher than that of the strains of *E. coli* and *Klebsiella aerogenes*.

Figure 2 shows the survival during 4 hr. of two strains of *Ps. aeruginosa*, one of which showed a high percentage survival at 2 hr. (26·8 %) while the other (strain F 15) showed a lower survival rate (5·8 % survivors at 2 hr.) in the previous experiment (see Table 2). The curves confirm the difference between the two organisms in survival on drying.

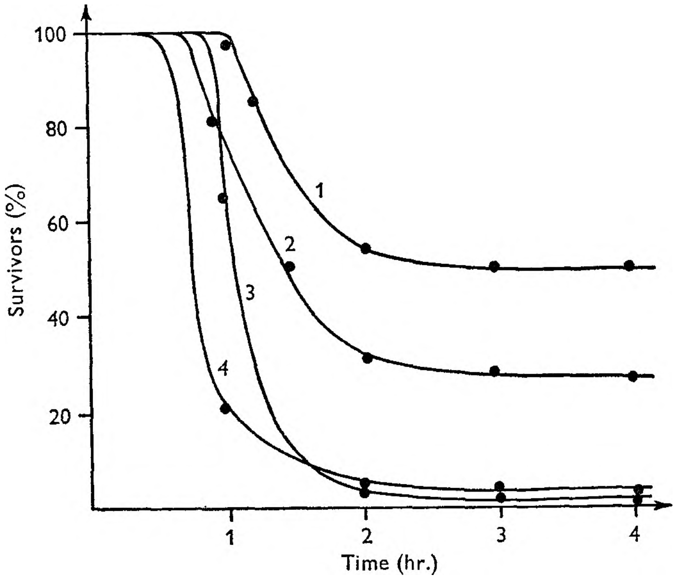

Fig. 1. Percentage of bacteria surviving on coverslips at intervals during and after the evaporation of suspending fluid (deionized water). 1, Micrococcus; 2, *Staphylococcus aureus*; 3, *Escherichia coli*; 4, *Klebsiella aerogenes*.

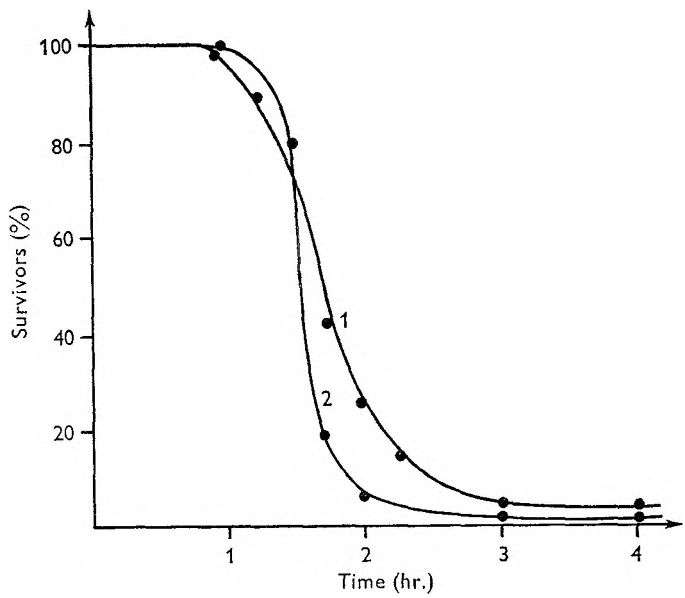

Fig. 2. Survival curves on drying (cf. Fig. 1) showing different proportions of survivors in two strains of *Pseudomonas aeruginosa*. 1, Survival curve for strain F 13; 2, survival curve for strain F 15.

Survival of Ps. aeruginosa *after drying, during periods of* 8 *weeks*

Of the eight strains examined, four showed survivors after 8 weeks' exposure on coverslips. These results were not related to place of isolation, phage type, or survival at 2 hr. In a repeat experiment, three out of the eight strains showed survivors, but only one strain showed survivors in both experiments after this period of exposure.

Survival of Ps. aeruginosa *at* 37° *and at* 4° *C.*

In the test made at 37° C. and relative humidity 20–25 %, the percentage of survivors was 0·24 %; at 4° C. and relative humidity 35–45 % the coverslips were still moist at 2 hr. and 35·5 % of the organisms remained viable. After 24 hr. there were 0·003 % survivors at 37° C. and 0·58 % survivors at 4° C.

THE SURVIVAL ON A SECOND DRYING OF BACTERIAL CELLS WHICH HAVE SURVIVED ONE DRYING

Materials and Methods

Bacterial strain

Ps. aeruginosa (strain F3) isolated from a human burn was used.

Preparation of suspensions and exposure to drying

A 20 ml. infusion broth culture of *Ps. aeruginosa* (strain F3) incubated at 37° C. for 24 hr. was centrifuged at 2000 rev./min. for 30 min., washed and resuspended in deionized water (approximately 3 ml.) to provide a concentrated suspension. Of this suspension, 0·03 ml. was then pipetted on to each of sixty coverslips; the drops were spread over the surface of the coverslips, left to dry and kept for 6 days in a closed cardboard box.

Estimation of the number of organisms surviving exposure on coverslips

After 6 days, organisms were extracted from ten coverslips by shaking with 5 ml. of deionized water in a Universal container, as described above, and a viable count was made.

On the seventh day the fifty remaining coverslips were extracted in deionized water. The suspension was concentrated by centrifugation to give approximately 2×10^7 viable organisms/ml., and this was used for a 4 hr. drying experiment as described above.

Results

Figure 3 shows the survival curve on drying of previously dried, resuspended bacteria, compared with that for suspensions of the same strain prepared from a fresh culture and from a culture of the once-dried organisms. Although all these suspensions show a rapid decrease in numbers followed by a period of more gradual decreases, these decreases were smaller and less rapid with the pre-dried organisms than with the suspensions prepared from cultures. A considerably higher proportion (approximately 15 % more) of the pre-dried organisms than of the organisms resuspended from culture remained viable when the numbers of survivors reached their constant level at the end of the experiment. When organisms which had been left to dry for a period of time, as described above, were harvested and grown overnight in broth, this new population tested for survival on drying showed a survival curve which was similar to that of the fresh suspensions of organisms.

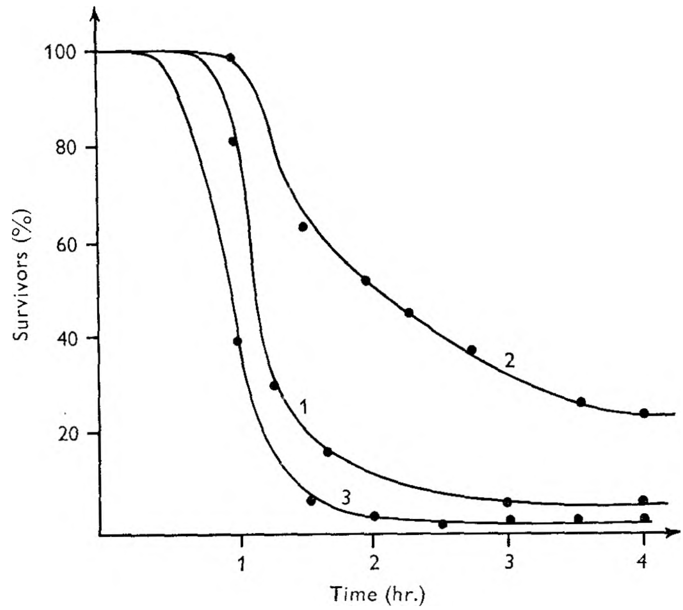

Fig. 3. Survival curves on drying of *Pseudomonas aeruginosa* cells before and after they had survived one exposure to drying, and of a culture of dried cells. 1, Survival curve for fresh suspensions of organisms; 2, survival curve for organisms previously dried; 3, survival curve, after subculture, for organisms previously dried.

SURVIVAL OF *PS. AERUGINOSA* AND *STAPH. AUREUS* IN WATER AND IN SOLUTIONS

Materials and Methods

Bacterial strains

Ps. aeruginosa (strains F 3 and F 16) and *Staph. aureus* (strains FS 2 and FS 6) were used to test survival in suspension for a limited period of time (6 hr.). Seven strains of *Ps. aeruginosa* (F 1, F 3, F 6, F 11, F 12, F 13, and F 15) and seven strains of *Staph. aureus* (FS 1–7) were selected for tests on survival in suspension during a period of several weeks.

Preparation of suspensions

Suspensions were prepared as described in the previous section, after washing with deionized water. Two drops (0·03 ml./drop) of freshly prepared suspensions were then added to 20 ml. amounts of sterile deionized water, physiological saline solution and Ringer's solution for tests of survival during 5 hr., and to the same amounts of deionized water, tap water and Ringer's solution for tests of survival during several weeks. The diluted suspensions were briefly agitated to disperse the bacteria.

Sampling and bacterial counts

In the first experiment 0·5 ml. samples were removed in duplicate immediately after the suspending fluids were inoculated, and from then at 15 min. or 30 min. intervals for the duration of the 5 hr. Viable counts were made on each of these samples.

In the second experiment, viable counts were made on 0·5 ml. samples removed at the start and at 24 hr. From then, loopfuls of the suspensions were removed twice weekly for 3 weeks, and at weekly intervals during periods of up to 25 weeks. These were spread on the surface of nutrient-agar plates and the presence or absence of growth recorded after a minimum of 24 hr. incubation at 37° C.

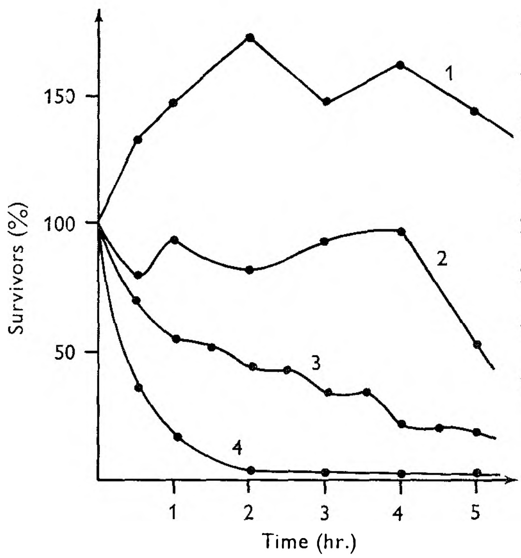

Fig. 4

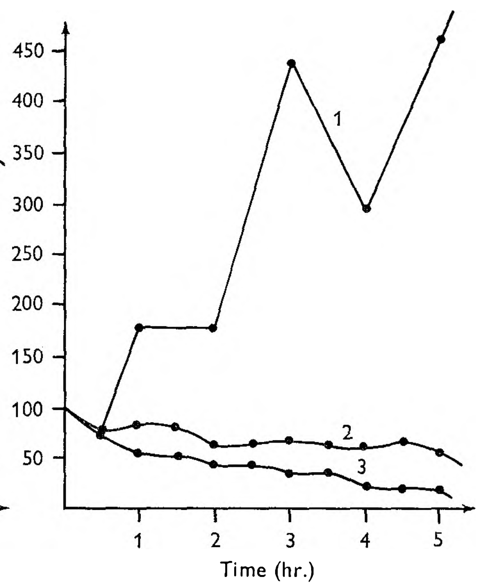

Fig. 5

Fig. 4. Survival curves for 5 hr. of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in deionized water. 1, *Staph. aureus*, strain FS 6; 2, *Staph. aureus* strain FS 2; 3, *Ps. aeruginosa* strain F 3; 4, *Ps. aeruginosa* strain F 16.

Fig. 5. Survival curves for 5 hr. of a strain of *Pseudomonas aeruginosa* in deionized water, physiological saline and Ringer's solution. 1, In Ringer's solution; 2, in saline; 3, in deionized water.

Results

Figure 4 shows the survival curves over a period of 5 hr. for *Staph. aureus* (strains FS2 and FS6) and *Ps. aeruginosa* (strains F3 and F16) suspended in deionized water. Both strains of *Staph. aureus* show a higher percentage survival than the strains of *Ps. aeruginosa*. During the first 2 hr. *Staph. aureus* (strain FS6) showed an increase (approximately 70%) over the number of organisms present initially, followed by a fluctuating decline in numbers. FS2, after an initial drop of 20% in the number of viable organisms present, showed a pattern of increase and decrease similar to that shown by FS6, though the number of viable organisms present during the experiment never exceeded the numbers present at the beginning; at 5 hr. approximately 52 % were still shown to be viable. *Ps. aeruginosa* (strain F 3) showed a steady decrease in the number of viable organisms, 18 % of those initially present still being found viable at 5 hr.; a more rapid decline in the number of survivors of strain F 16 occurred during the first 2 hr., but the numbers then stayed constant at 1·5 % of those initially present.

Figure 5 shows the survival curves over a period of 5 hr. for *P. aeruginosa* (strain F 3) suspended in saline, deionized water and Ringer's solution. Organisms suspended in saline showed an initial decline in numbers of viable organisms, followed by a more gradual fluctuating decline, to approximately 54 % of the organisms initially present at the end of the 5 hr. period. In deionized water the pattern of decline was similar, but with greater loss, approximately 18 % of the organisms initially present surviving after 5 hr. By contrast, in Ringer's solution the number of surviving organisms fell during the first 30 min., but then rose by irregular stages to approximately 460 % of the initial counts after 5 hr.

In the experiment with strains left in suspension for several weeks (see Tables 4 and 5) viable counts of *Ps. aeruginosa* were considerably reduced after 24 hr. in deionized water and tap water, but much greater reductions occurred with *Staph. aureus*; in deionized water, strains of *Ps. aeruginosa* showed between 1·9 and 12 % of survivors, but with strains of *Staph. aureus* the proportion of survivors in deionized water was between 0·003 % and 0·5 %. In tap water, survivors of all strains of *Ps. aeruginosa* were fewer than 1 %; no strain of *Staph. aureus* showed any survivors after 24 hr. in tap water, or on subsequent samplings from tap water or deionized water.

In Ringer's solution, at the 24 hr. and 48 hr. samplings all strains of *Ps. aeruginosa* gave confluent growth from the dilution used for making viable counts on the initial sample, indicating an increase in numbers. With *Staph. aureus*, fewer than 1 % of the numbers present at the start were present after 24 hr., and after 48 hr. viable organisms could only be detected in three out of the seven strains. No viable organisms were found on subsequent samplings.

After 25 weeks, viable organisms were found in suspensions of all strains of *Ps. aeruginosa* in Ringer's solution and tap water; viable organisms could be detected in four out of the seven strains in deionized water.

DISCUSSION

As in the earlier studies of Lowbury & Fox (1953), the work reported here showed that a smaller proportion of Gram-negative bacilli than of Gram-positive cocci survived after the atmospheric drying of suspensions. Variations in the pattern of survival were found, on replicate testing of strains with different death rates, to be consistent. It was also found that strains of *Ps. aeruginosa* isolated from the dust of floors had, in most cases, a lower death-rate on drying than strains isolated from patients. An even larger difference was found between the death-rate on drying of *Ps. aeruginosa* cells from natural sources and the much reduced death-rate of *Ps. aeruginosa* cells resuspended in distilled water

Table 4. *Survival of* Staphylococcus aureus *in suspension*

(+ = presence of growth; − = absence of growth.)

			Survival in								
			Ringer's solution			Deionized water			Tap water		
Strain	Phage type	Antibiotic resistance*	24 hr.	48 hr.	96 hr.	24 hr.	48 hr.	96 hr.	24 hr.	48 hr.	96 hr.
FS 1	52A/80	P.T	0·6 %	+	−	−	−	−	−	−	−
FS 2	3C	Sens.	−	−	−	−	−	−	−	−	−
FS 3	80/81 (1000 × R.T.D.)	T.N.Ne	0·9 %	+	−	0·1 %	−	−	−	−	−
FS 4	29/52/52A/80	P	0·15 %	−	−	−	−	−	−	−	−
FS 5	29/52	Sens.	0·8 %	+	−	0·003 %	−	−	−	−	−
FS 6	29/77	P.S.T.C.E.N.Ne	0·25 %	−	−	0·5 %	−	−	−	−	−
FS 7	75/77	S.T.E.Ne	−	−	−	0·005 %	−	−	−	−	−

* P = penicillin, S = streptomycin, T = tetracycline, C = chloramphenicol, E = erythromycin, N = novobiocin, Ne = neomycin, sens. = sensitive to all antibiotics tested.

Table 5. *Survival of* Pseudomonas aeruginosa *in suspension*

(+ = presence of growth; − = absence of growth.)

			Survival in									
			Ringer's solution		Deionized water						Tap water	
Strain	Phage type	Source	24 hr.	25 weeks	24 hr.	5 weeks	6 weeks	16 weeks	17 weeks	25 weeks	24 hr.	25 weeks
F 1	21/68/F 7	Burn	C.G.*	+	6·2 %	+	+	+	+	+	0·68 %	+
F 3		Burn	C.G.	+	8·5 %	+	+	+	+	+	0·19 %	+
F 6	68/73/109/119x	Wound B	C.G.	+	11·2 %	+	−	−	−	−	0·27 %	+
F 11	F 8	Dust (Burns Ward)	C.G.	+	12·0 %	+	+	+	+	+	0·2 %	+
F 12	21/68	Dust (Burns Ward)	C.G.	+	5·7 %	+	+	+	−	−	0·2 %	+
F 13	7/31/352	Ward floor	C.G.	+	2·2 %	+	−	−	−	−	0·12 %	+
F 15	68/73/109/119x/+	Ward floor	C.G.	+	1·9 %	+	+	+	+	+	0·57 %	+

* Confluent growth.

after they had survived one drying. These dried organisms, however, reverted to their original survival pattern on subculture; the reduced death-rate of the cells which had survived one drying was, presumably, an aspect of the greater viability of the survivors than of the great majority of cells in the original suspensions.

Differences in viability on drying of strains from infections in a ward would be expected to lead to some selection in dust of the strains which survive better after exposure to drying. This was supported to some extent by the different phage types of *Ps. aeruginosa* found on the floor in a burns ward and in the burns of patients occupying the ward. Though *Ps. aeruginosa* is not commonly found in air samples, it is usually found in the dust of burn wards, and dust-borne or air-borne *Ps. aeruginosa* can be an important source of infection in burns (Lowbury & Fox, 1954; Lowbury, 1954; Hurst & Sutter, 1966).

The contrasted pattern of survival of *Staph. aureus* on the one hand and of *Ps. aeruginosa* on the other are well illustrated in this study. The staphylococci not only survived drying better than the strains of pseudomonas, but they were also shown to survive less well than pseudomonas in deionized water. There were some unexpected observations. *Staph. aureus* showed better survival in deionized water during the first 5 hr. than *Ps. aeruginosa*, which showed a large reduction in numbers during that period, but then, unlike the staphylococcus, persisted in small numbers for several weeks. In Ringer's solution, on the other hand, a strain of *Ps. aeruginosa* multiplied appreciably during the first 5 hr. and also survived for many weeks.

The relevance of these findings to the epidemiology of infection appears obvious, and they are consistent with the observed tendency of *Ps. aeruginosa* to be transferred in solutions and in moist vectors, while *Staph. aureus* is more likely than *Ps. aeruginosa* to be transferred by air. These generalizations must, however, be qualified in the light of other evidence from laboratory and field study. For example, strains of staphylococci vary greatly in their death-rate subsequent to drying, and some strains die out when dry almost as quickly as the Gram-negative rods; this is consistent with the rapid elimination of a *Staph. aureus* from a ward after the departure of a patient carrying staphylococci of the same type (Skaliy & Sciple, 1964), and from a wall contaminated by the fingers of a heavy carrier (Ayliffe, Collins & Lowbury, 1967).

Not only the viability but also the numbers of bacteria in the environment and their access to the patient will determine the role of environmental contaminants in causing infection. For example, *Staph. aureus* is very common in floor dust, but the small amount of redispersal of these organisms from the floor into the air makes their transmission by this route improbable (Ayliffe *et al.* 1967); the more likely sources of *Staph. aureus* are by direct contact transfer from carriers or by direct airborne contamination from heavy dispersers of the organisms. *Ps. aeruginosa*, unlike *Staph. aureus*, is rarely carried in large numbers by normal subjects, but in fluids it not only survives better than the staphylococcus but may multiply in water with few nutrient additives. This capacity may be important not only in making contaminated eyedrops and other solutions an important source of infection, but also, perhaps, in allowing the multiplication of *Ps. aeruginosa* from very small inocula on sloughs, in the aqueous humour (see Crompton, 1962), and in the cerebrospinal fluid. The same potentiality of growth from small inocula makes it possible for *Ps. aeruginosa* to be transferred in airborne dust and on the hands of nurses, where the numbers of the organism, if present, are usually very small, as well as in the more commonly recognized fluid vectors.

SUMMARY

Suspensions of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella aerogenes* and micrococci were tested for survival on drying and after drying in the atmosphere. The proportion of Gram-negative bacilli that died during drying was greater than that of the Gram-positive cocci, but survival after drying was similar in the two groups of organisms. There were considerable differences in the death-rates on drying of different strains of bacteria, which were consistent on replicate sampling.

A suspension of *Ps. aeruginosa* prepared from cells which had survived one drying showed a considerably higher proportion of survivors on a second drying; suspensions prepared from subcultures of the dried cells, however, showed a death-rate on drying which was similar to that of the original culture.

Strains of *Ps. aeruginosa* isolated from floor dust showed a significantly higher proportion of survivors immediately after drying than strains from patients, but the proportion of survivors after 24 hr. of exposure to the atmosphere was approximately the same in the two groups.

Tests were made for survival of *Ps. aeruginosa* and *Staph. aureus* in deionized water, tap water, physiological saline and Ringer's solution. In deionized water, *Ps. aeruginosa* showed a rapid initial loss but some survival for several weeks; *Staph. aureus*, on the other hand, showed an initial increase in numbers but no survivors after 48 hr. In Ringer's solution all strains of *Ps. aeruginosa* multiplied rapidly and survived for many weeks; *Staph. aureus* died rapidly and no strain could be detected after 4 days.

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Growth of rubella virus in human embryonic organ cultures

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INTRODUCTION

Organ cultures of foetal trachea and nasal epithelium have been used for the growth of viruses which cause acute respiratory infection (Hoorn & Tyrrell, 1965; Tyrrell & Bynoe, 1965, 1966; MacIntosh *et al.* 1967). This technique has been applied to the growth of rubella virus, since this virus can be isolated from the nasopharynx of both patients with rubella and infants with the rubella syndrome.

MATERIALS AND METHODS

Virus

Two strains of rubella virus were used, Judith (McCarthy & Taylor-Robinson, 1965) and Thomas, the latter having been isolated at St Thomas's Hospital in 1966 from an infant with congenital rubella. Judith was propagated in both RK 13 and BHK-21 cell cultures. Since its original isolation, Thomas had been passed eight times in RK 13 and BHK-21 cell cultures. Titres of virus used for inoculation varied from < 10 TCD 50/ml. to $10^{6\cdot 5}$ TCD 50/ml.

Organ cultures

Embryonic trachea, nasal mucosa and, on some occasions, pharynx, larynx, skin and brain were obtained from foetuses aged 15–28 weeks. These were dissected within 4–22 hr. of hysterotomy according to the method of Hoorn & Tyrrell (1965). Two or three pieces of trachea or one piece of nasal epithelium were placed in each Petri dish (Nunc brand) with 1·25 ml. medium for trachea and skin cultures and 2–3 ml. for nasal epithelium, pharynx, larynx and brain. The pieces of pharynx, larynx, skin and brain were cut into pieces 5–10 mm square.

Initially medium consisted of Medium 199 (Burroughs Wellcome and Co.), containing sodium bicarbonate 0·088 %, bovine plasma albumin 0·2 %, penicillin 200 units/ml., and streptomycin 200 μg/ml. Cultures were incubated at 36° C. in an atmosphere of 4 % CO_2 in air. In later experiments Leibovitz L 15 medium (Flow Laboratories Ltd.) containing foetal calf serum 2 %, glutamine 0·029 %, penicillin 200 units/ml. and streptomycin 200 μg./ml. was found to be satisfactory, since cultures could be incubated in a humidified box without added CO_2.

Organ cultures were inoculated 1–3 days after preparation. After removing medium, virus was dropped on to the pieces of organ culture. The maximum volume of virus inoculated was equal to the volume of medium initially in the Petri dish. Cultures were then replaced at 36° C. for approximately 6 hr. to allow adsorption of virus, after which the medium was changed. Control cultures were either left uninoculated or were inoculated with an equal volume of tissue culture fluid which contained no virus. In some experiments a virus-control Petri dish was included containing virus, but no organ cultures. The inoculum in this dish was left unchanged when we wished to test the survival time of the inoculated virus.

In growth curve experiments the medium from each of the Petri dishes was *harvested daily, 'snap' frozen and stored at* -70° *C., after which fresh medium* was added. During other experiments the medium was changed at intervals of 2 or 3 days. Cultures were examined daily for ciliary activity.

Virus titrations

The harvested fluids from each experiment including those from all control cultures were titrated in parallel in RK 13 cell cultures (Best & Banatvala, 1967). In order to detect the presence of intracellular virus, cultures of trachea and nasal epithelium were inoculated with rubella virus, uninoculated controls being included in the test. Cultures of trachea were tested on days 4 and 12, and cultures of nasal epithelium on days 3 and 11; the trachea and nasal epithelium were obtained from different foetuses in these experiments. The pieces of tissue were weighed, washed twice with PBS (Dulbecco Solution A), ground with a pestle and mortar and a 1 % (w/v) suspension prepared. This was then sonicated for 2 min. using an MSE ultrasonic unit. These suspensions were stored at -70° C. until they could be titrated.

Histological studies

Pieces of tissue were fixed in Bouin's fluid and embedded in paraffin wax 13 days after inoculation. Sections were stained with haematoxylin and eosin and were examined for structural changes.

RESULTS

Twenty-seven foetuses were used for the preparation of trachea and nasal epithelium organ cultures. Pharynx was used in three experiments, larynx in two, and both skin and brain in one experiment only. Material from two foetuses was discarded because of contamination and no virus was recovered from a further seven, but it was shown subsequently that insufficient virus had been inoculated. Organ cultures from the remaining eighteen foetuses supported the growth of rubella virus.

Titration of tracheal organ culture fluids from two typical experiments produced the growth curves shown in Fig. 1. No virus was detected in the organ culture fluids for 48 hr. after inoculation, after which virus titres rose from $10^{1\cdot5}$ to $10^{2\cdot2}$/ml., being maintained at approximately this level until the experiments were discontinued on day 11 and day 12. However, further experiments revealed that titres could be maintained at this level for at least 34 days following inoculation. Virus was not isolated from uninoculated control cultures, but some virus was recovered from the virus control dishes during the first 48 hr. after inoculation when the medium was not changed at 6 hr.

Table 1 shows that intracellular and organ-culture fluid virus titres were similar in both trachea and nasal epithelium when tested on two occasions.

Table 2 shows details of organ-culture experiments in which high titres were obtained in the organ culture fluid. Virus titres obtained in organ culture fluids tended to vary with the titre of virus in the original inoculum. The highest titre was obtained in nasal epithelium on day 9 in Expt. 22 ($10^{3\cdot7}$TCD 50/ml.), when 2·5 ml. of virus at a titre of $10^{6\cdot5}$/ml. was inoculated. Although virus of a similar titre was inoculated in Expt. 12, it failed to yield as high a titre; however, only

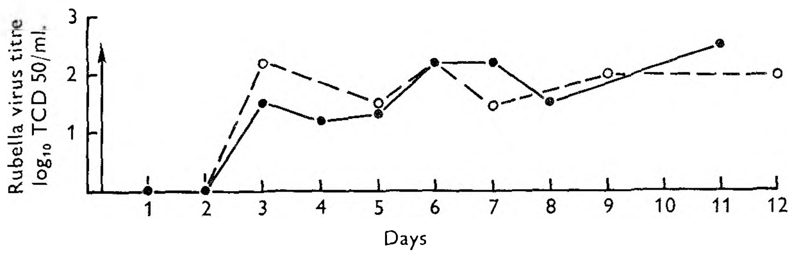

Fig. 1. Two typical growth-curve experiments, showing the multiplication of rubella virus in organ cultures of human embryonic trachea. The arrow indicates the titre of virus inoculated.

Table 1. *Comparison of intracellular and organ-culture fluid virus titres*

	Days after inoculation	Titre of rubella virus ($\log_{10}$TCD 50/ml.) Fluid	Tissue*
Trachea	4	1·2	1·2
	12	2·2	2·5
Nasal	3	1·7	2·2
	18	2·2	2·7

* 1 % (w/v) suspension prepared from organ culture tissue.

1·0 ml. of tissue culture fluid was inoculated and this was allowed to adsorb for only 3 hr. No virus was recovered in experiments in which < 10 TCD 50/ml. of virus was inoculated. Rubella virus usually grew as well in pharynx, larynx and skin as in trachea and nasal epithelium. The only experiment in which all these organs were used from the same foetus produced the virus titres shown in Table 3. In the only experiment in which brain was included, rubella virus at a titre of $10^{1\cdot2}$TCD 50/ml. was recovered on day 8.

Rubella virus could be passed successfully in organ cultures. After two passages, Judith produced a titre of 10^3/ml. (Expt. 13), but this did not increase after two further passages (Expt. 18). Thomas was passed twice to produce a final titre of $10^{2\cdot4}$/ml.

Ciliary activity was maintained throughout in inoculated cultures of trachea, nasal epithelium, pharynx and larynx in all experiments. Histological studies

Table 2. *Details of organ-culture experiments in which high titres of rubella virus were obtained*

Expt. no.	Age of foetus (weeks)	Virus inoculum: Strain of virus	Virus inoculum: Titre (log_{10} TCD 50/ml.)	Volume inoculated: Trachea	Volume inoculated: NE*	Highest titre in organ culture fluid, Trachea: Titre (log_{10} TCD 50/ml.)	Trachea: Day obtained	Nasal epithelium: Titre (log_{10} TCD 50/ml.)	Nasal epithelium: Day obtained
				1st passage					
12	22	Judith (BHK)	6·0	1	1	1·5	4 and 23	1·4	4 and 23
19	16	Thomas (BHK)	5·1	1·25	2·5	2·4	11	3·3	11
22	23	Judith (BHK)	6·5	1·25	2·5	2·5	12	3·7	9
				2nd passage					
13	18	Judith (o.c. fluid)	2·0	0·6	0·75	3	21–27	1·4	14–27
20	17	Thomas (o.c. fluid)	2·0	1·25	2	< 1·0	3–13	2·4	13
				3rd passage					
16†	16	Judith (o.c. fluid)	3·0	0·4	0·4	3	9	2·4	9
				4th passage					
18	17	Judith (o.c. fluid)	1·5	1·25	2·5	3	18	2·4	34

* Nasal epithelium. † Larynx used instead of nasal epithelium in this experiment.

revealed no differences between control and inoculated sections of organ cultures. Cilia were clearly seen to be present and no degenerative changes or inclusion bodies were seen.

Table 3. *Results of organ culture fluid titrations using cultures derived from the same foetus*

Days after inoculation	Titre of rubella virus. $\log_{10}$ TCD 50/ml.			
	Trachea	Nasal epithelium	Pharynx	Skin
5	1·5	3·4	1·5	2·5
7	0·5	3·4	0·5	2·2
9	0·5	3·7	0·5	2·2
12	2·5	3·4	—*	1·0

* Not tested.

DISCUSSION

These *in vitro* experiments show that rubella virus replicates consistently in human tissue derived from embryonic trachea and nasal mucosa and can persist in these cultures for up to 34 days without apparent damage to the ciliated epithelium. This contrasts with the effects of some viruses causing acute respiratory infection, which destroy the cilia and damage the epithelial surface (Tyrrell & Hoorn, 1966). However, electron-microscopic studies conducted on sections stained with osmium tetroxide derived from some of our organ cultures showed inclusion bodies containing vesicular structures 400–1200 Å in diameter in the mucous secreting cells and virus-like particles at the cell surface (Kistler, Best, Banatvala & Töndury, 1967). These particles were 400–700 Å in diameter and were morphologically similar to those described in other recent reports on the structure of rubella virus (Best *et al.* 1967; Holmes & Warburton, 1967).

Although congenital malformation is associated with maternal infection by rubella virus during the first trimester of pregnancy (Gregg, 1941; Manson, Logan & Loy, 1960; Lundström, 1952, 1962), these experiments show that organs from foetuses aged 15–28 weeks are capable of supporting the growth of rubella virus *in vitro*. It was not possible to study the features of infection in organ cultures derived from foetuses aged less than 15 weeks, since these are not generally delivered by hysterotomy. Whether the placenta forms an effective barrier to virus entering the foetal circulation after the first trimester or whether foetal infection occurs without causing any persisting damage has not been clearly determined. That virus may cross the placenta in late pregnancy is supported by a report of a case in which rubella occurred during the last week of pregnancy. An apparently healthy infant was born at term but virus was isolated from his blood on the day of delivery, and from the pharynx 3 days later (Banatvala *et al.* 1965).

Although a limited number of experiments were conducted with embryonic organs other than trachea and nasal epithelium, the replication of virus in pharynx, larynx, skin and brain suggests that many foetal organs will support the growth of rubella virus *in vitro*. In addition growth of rubella virus in organ cultures of rabbit and hamster lung has been reported by Oxford & Schild (1967). These findings are consistent with the recovery of virus from a variety of different tissues derived from infants with the rubella syndrome (American Pediatric Society and Society for Pediatric Research, 1965). It was not possible to conduct histological studies on organ cultures other than trachea and nasal epithelium in this series, but it is hoped to conduct further studies on these and additional infected organs.

The only other report of the multiplication of rubella virus in human organ culture is by Albanese & Romano (1967), who infected human embryonic and rabbit trachea with the Judith strain of rubella virus and reported loss of ciliary activity and degenerative changes in the epithelium, loss of ciliary activity beginning 24 hr. or less after inoculation. However, they recovered virus from organ culture fluids on one occasion only, and failed to recover virus from both organ culture tissue and the remaining daily harvests of fluid. Using a similar or greater virus inoculum we have consistently been able to recover virus from the organ culture fluid and tissues, without observing any loss of ciliary activity. Oxford & Schild (1967) showed that ferret trachea would support the growth of rubella virus without damage to the cilia or epithelial structure.

Since rubella virus has been shown to replicate successfully in organ cultures of human embryonic tissues, this may provide a convenient experimental system for studying its pathogenesis, as the cells in these cultures unlike cells found in monolayer cell cultures are similar in structure and physiology to those of the intact human host.

SUMMARY

Two strains of rubella virus multiplied in organ cultures of human embryonic trachea, nasal epithelium, pharynx, larynx, skin and brain derived from foetuses aged 15–28 weeks. Growth curve experiments conducted on cultures of nasal epithelium and trachea showed that virus appeared in the culture fluid 72 hr. after inoculation and thereafter rose to titres varying from 10 to $10^{3\cdot7}$TCD 50/ml. These titres persisted for periods up to 34 days after inoculation. Intracellular and organ culture fluid virus titres were shown to be similar in specimens tested in both the early and late stages of the growth curve. No degenerative changes or loss of ciliary activity was observed in these cultures.

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Studies on the LS antigen of vaccinia virus

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The LS antigen of vaccinia virus was described first by Craigie & Wishart (1936*a*). It was a material which could be eluted from purified virus particles and occurred also in virus-free extracts of vaccinia-infected rabbit dermis (soluble antigen). The substance was not detectable in uninfected rabbit tissue. It was called LS because it possessed two different serological specificities. One specificity (L) was 'heat labile' and destroyed by heating soluble antigen to 56° C. for 45 min.; the other specificity (S) was 'heat stable' and not altered by this treatment. The two specificities were thought to be associated with a single substance because either specific anti-L antibody or specific anti-S antibody precipitated both L and S serological activities from soluble antigen. Specific anti-L antibody was prepared by inoculation of rabbits with partially purified LS and absorption of the resulting antisera with LS heated to destroy L. Specific anti-S antibody was prepared by inoculation of rabbits with heated LS (Craigie & Wishart, 1936*b*).

Craigie & Wishart (1938) stated that under certain conditions LS antigen might dissociate and occur in soluble antigen as separate L and S substances. Materials with the serological properties of S only were obtained from vaccinia-infected rabbit tissue by Smith (1932), Ch'en (1934) and Parker & Rivers (1937), but a material with the serological properties of L only was not described at this time. A substance which was electrophoretically and ultracentrifugally homogeneous and possessed the serological properties of LS antigen was isolated from vaccinia-infected rabbit dermis by Shedlovsky & Smadel (1942). Smadel & Shedlovsky (1942) suggested that by suitable treatment the purified LS antigen might be converted to various serological states which ranged from L-inactive, S-active to L-active, S-inactive.

More recently gel-diffusion studies have shown many distinct virus-specific serological entities in extracts of vaccinia-infected tissues. In immunodiffusion tests on such extracts Gispen (1955) found up to six line pattern components (l.p.c.), Rondle & Dumbell (1962) up to 9 l.p.c., and Westwood *et. al.* (1965) up to 17 l.p.c. Because of this complexity Westwood *et al.* suggested that the original concept of a single LS antigen was likely to have been an expression of the collective behaviour of a number of antigens. Joklik (1966) also stated that the LS antigen was probably not a singular molecular species. Certainly Nicoli & Jolibois (1964) were unable to find an LS-specific substance in extracts of unspecified tissues infected with vaccinia virus. They detected seven virus-specific substances in the soluble antigen, found materials with properties originally ascribed to L and S, but separated these by fractionation on DEAE-cellulose. In addition, Cohen & Wilcox (1966) examined an LS preparation made from vaccinia-infected KB or L cells. The material which was obtained by precipitation of soluble antigen at pH 4·6 represented 6 % by weight of the protein originally present and possessed 75 % of the original complement-fixing activity. It gave two zones in polyacrylamide gel tests, 2 l.p.c. in immunoelectrophoresis tests, and 3 l.p.c. in immunodiffusion tests. The heat stability of the material was not reported. Other results were obtained by Marquardt, Holm & Lycke (1965*a*, *b*). They found eight virus-specific l.p.c. in immunodiffusion tests with extracts of vaccinia-infected rabbit dermis. Some of the l.p.c. (three or more) were designated as 'e–f complex'. The complex passed unchanged through a column of DEAE-cellulose, being eluted by 0·1–0·2 M sodium chloride. It resembled LS in that it precipitated at pH 4·8 and its serological activity was partly destroyed by trypsin, partly heat labile, and partly heat stable.

We studied LS antigen initially to relate it serologically to the 9 l.p.c. found by Rondle & Dumbell (1962) in immunodiffusion tests on extracts of vaccinia-infected rabbit tissue. We also attempted to isolate LS from vaccinia-infected sheep dermis and to study the properties of S-antigen. Our results tend to reconcile the conflicting views on LS and lead to interesting speculations on vaccinia virus replication in different hosts.

MATERIALS AND METHODS

Virus

The 'Lister' strain of vaccinia virus was used throughout this work.

Soluble antigen

Virus-free extracts of vaccinia-infected rabbit dermis and chick chorio-allantois were prepared as described by Rondle & Dumbell (1962). Samples of soluble antigen from vaccinia-infected sheep dermis were a gift from the Lister Institute, Elstree, through the good offices of Professor C. Kaplan.

Preparation of LS from rabbit soluble antigen

Seven preparations of LS were made from extracts of vaccinia-infected rabbit dermis by a method which differed little from that described by Shedlovsky & Smadel (1942). In the largest experiment soluble antigen (341 ml.) from sixteen rabbits was filtered through a Seitz EK filter, concentrated by pervaporation in 'Visking' dialysis tubing to 41 ml. and dialysed 16 hr. at 4° C. against 0·9 % saline. Pervaporation was done in a cold room at 4° C. by suspending a dialysis sac filled with soluble antigen in a fan-induced draught. The dialysis sac containing the concentrated soluble antigen was agitated constantly for 6·5 hr. at 4° C. in

1 l. of 0·06 M sodium acetate–acetic acid buffer, pH 4·6. The contents of the sac were then centrifuged for 10 min. at 3000 rev./min. and the supernatant fluid discarded. The precipitate was washed twice with acetate buffer, pH 4·5, suspended in 20 ml. of 0·05 M phosphate–phosphate buffer pH 6·3 and dialysed against this buffer for 16 hr. at 4° C. Soluble and insoluble materials were separated by centrifugation for 10 min. at 3000 rev./min. The insoluble precipitate was extracted twice with phosphate buffer, pH 6·3, and the clarified extracts added to the bulk of the soluble material. The soluble material was then dialysed against water until free from salt and dried from the frozen state. The material obtained corresponded to 'Fraction B' of Shedlovsky & Smadel (1942), and was considered to be LS. In the experiment described the 23 mg. of LS obtained represented a yield of 2 % in terms of the indiffusible material present in the starting material.

Preparation of S from sheep soluble antigen

An attempt was made to prepare from sheep soluble antigen a material analogous to the S substance prepared by Parker & Rivers (1937) from rabbit soluble antigen. Sheep soluble antigen was dialysed against water until free from salt and dried from the frozen state. Some of the material (5 g.) was dissolved in 50 ml. 0·004 M phosphate–citrate buffer, pH 7·0, and heated at 98° C. for 5 min. The precipitate was discarded and the supernatant fractionally precipitated by additions of saturated ammonium sulphate. The material precipitating between 25 % and 50 % saturation with ammonium sulphate was preserved, dissolved in water, and obtained free from ammonium sulphate and dissolved in 0·01 M phosphate–phosphate buffer, pH 7·5, by use of a column of Sephadex G_{25} (Pharmacia Ltd., Uppsala, Sweden). The fractional precipitation with ammonium sulphate was repeated. To the dissolved precipitate nine volumes of ethanol were added at 4° C. The precipitate was taken up in 0·01 M phosphate buffer pH 7·5, residual ethanol removed on a column of Sephadex G_{25} and 5 ml. of M sodium acetate–acetic acid buffer, pH 4·5, added to the 35 ml. of solution obtained. As measured with a glass electrode the solution was pH 4·55. After standing overnight at 4° C. the precipitate was collected and dissolved in 0·01 M phosphate buffer, pH 7·2. Insoluble material was discarded and the solution adjusted to pH 7·8 with 0·1 N sodium hydroxide. The solution was heated to the apparent boiling point for 3 min. but no precipitate formed. Acetic acid (N) was then added to pH 6·0 and the solution again heated to the apparent boiling point for 3 min. Insoluble material was removed by centrifugation for 10 min. at 3000 rev./min., the supernatant dialysed against distilled water until free from salt and dried from the frozen state. The weight of material obtained (6·6 mg.) represented a yield of 0·013 %. In this paper the material obtained is referred to as S(PR).

Preparation of L and S from sheep soluble antigen

Five attempts were made to prepare LS from vaccinia-infected sheep dermis by the acid precipitation technique of Shedlovsky & Smadel (1942). Each attempt was made on a fresh sample of sheep soluble antigen and each attempt was unsuccessful. The precipitate obtained had no heat-labile serologically active components and precipitated poorly with antisera. Further attempts were made to fractionate sheep soluble antigen by using DEAE-cellulose (Kodak Ltd., Kirby Trading Estate, Liverpool). Soluble antigen was dialysed to equilibrium against 0·05 M phosphate buffer pH 7·4 and absorbed on a column of DEAE-cellulose. Fractionation was then attempted by flushing the column with buffer solutions containing stepwise increases in NaCl concentration. The results are described in full by Williamson (1963). With antisera prepared as described below the eluate at 0·137 M-NaCl reacted serologically as a specific L substance and the eluate at 4 M-NaCl reacted serologically as a specific S substance. Further work on the latter material gave a physically and serologically homogeneous S substance (Williamson & Rondle, 1964).

Disk electrophoresis

This technique measures the electrophoretic mobility of materials in columns of polyacrylamide gel and was carried out as described by Ornstein & Davies (1961).

Analytical ultracentrifugation

Through the courtesy of Dr W. Taylor, Department of Chemical Pathology, The United Liverpool Hospitals, samples of the S preparation were examined in a Spinco Model E ultracentrifuge by Dr A. Neill Wright of the Thornton Research Centre.

Ultraviolet absorption spectra

These were determined using a Hilger 'Uvispek' spectrophotometer.

The antisera used

The anti-vaccinia sera used in this paper were prepared with rabbit-grown materials as described by Rondle & Dumbell (1962). The specific anti-S serum was one of the sera prepared by Harris, Harrison & Rondle (1963) by repeated intravenous injection of rabbits with virus-free extracts of vaccinia-soluble antigen prepared from infected chick chorioallantois. Before injection the soluble antigen was heated to 60° C. for 45 min. in a sealed ampoule submerged in a water bath. A 'semi-specific' anti-L serum was prepared from an anti-vaccinia serum. Samples of serum were taken and mixed with different amounts of S(PR). The mixtures were tested for their ability to react with S(PR) in gel-diffusion experiments. The mixture which contained the least amount of S(PR) but which failed to react with S(PR) was regarded as a 'semi-specific' anti-L serum. From the results of Rondle & Dumbell (1962) such a mixture must have contained many different anti-vaccinia antibodies, but it was considered to contain no anti-S.

Gel diffusion tests

The final concentrations of materials in the gel used were: agar 1 %; 0·05 *I* phosphate buffer, pH 7·2; merthiolate (a gift from Eli Lilly and Co.) 1/10,000. On microscope slides a gel thickness of 1 mm. was employed and wells were cut in isometric patterns 4 mm. in diam. with centres 7 mm. apart. In Petri dishes a gel thickness of 3 mm. was used and wells were 9 mm. diam. with centres 14 mm. apart.

Antisera were used undiluted. Unless stated otherwise the LS and S(PR) preparations were used at a concentration of 0·5 mg./ml., the S preparation at 5 mg./ml.

RESULTS

Physical examination of materials

Two of the rabbit LS preparations tested gave one band when subjected to electrophoresis in polyacrylamide gel. The distance moved by this band divided by the distance moved by the bromophenol blue marker band (relative distance or R_d value) was 0·95. The other rabbit LS preparations gave an intense band at $R_d = 0·95$ and a second very weak band at $R_d = 0·56$.

The sheep S preparation gave a single band at $R_d = 0·92$ when tested, but the preparations of sheep S(PR) and sheep L both gave two bands. The position of these bands did not correspond with each other or with the single band given by sheep S, or with the one or two bands given by rabbit LS.

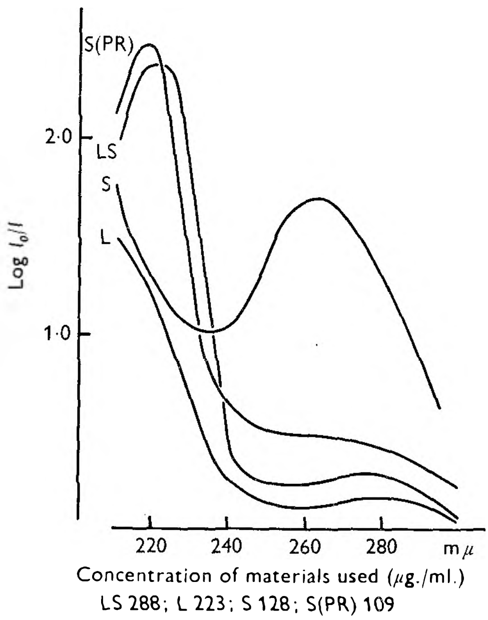

Fig. 1. Ultraviolet absorption spectra of LS, L, S, S(PR).

It was possible to test only the sheep S preparation in an analytical ultracentrifuge. As stated by Williamson & Rondle (1964) it gave a single sharp peak and from the data a molecular weight of approximately 31,000 was calculated.

These results show that the sheep S(PR) and L preparations were physically heterogeneous; they suggest that most of the rabbit LS preparations contained a major and a minor component; they support the view that the sheep S preparation was homogeneous and electrophoretically different from the rabbit LS and sheep S(PR) preparations.

Ultraviolet-light absorption curves for the various preparations are shown in Fig. 1. They indicate that the rabbit LS, sheep S(PR), and sheep L preparations contain protein but little nucleic acid while sheep S contains much nucleic acid. Calculated from a standard curve of commercial calf thymus nucleic acid, S(PR) contains less than 6 % and LS and L less than 2 % nucleic acid, while S contains approximately 70 % of it. Chemical studies have shown that the nucleic acid of sheep S is DNA (Williamson & Rondle, 1964).

Gel diffusion study of materials

Results obtained with those preparations of rabbit LS which gave only one band in polyacrylamide electrophoresis will be given first.

Freshly prepared solutions (30 μg./ml. to 1 mg./ml.) of fresh preparations of LS from vaccinia-infected rabbit dermis frequently gave two lines in a gel diffusion test when examined against anti-vaccinia rabbit sera (AVS). The phenomenon is shown in Pl. 1, fig. 1, which shows also that one l.p.c. of LS disappeared on conversion of LS to LS(H) by heating LS at 56° C. for 45 min. and that the l.p.c. which was stable to heat treatment was serologically identical with sheep S(PR).

This result could have been obtained from a mixture possessing discrete L and S serological specificities. This is unlikely, however, since, as shown in Pl. 1, fig. 2, mixing rabbit LS with a specific anti-S serum resulted in complete loss of reaction with an anti-vaccinia serum which was known to contain anti-L. The specificity of the anti-S serum is demonstrated in Pl. 1, fig. 3, which shows that the anti-S serum gave only one line of precipitation when tested against vaccinia-soluble antigen prepared from infected rabbit dermis. The soluble antigen (V) used gave a complex line pattern when tested against an anti-vaccinia serum. The experiment shown in Pl. 2, fig. 11, shows additionally that the anti-S serum did not react with a preparation of a sheep L substance (see below).

A further indication that the two specificities were closely associated was that they could be absorbed on DEAE-cellulose equilibrated at pH 7·4 with phosphate–phosphate buffer $I = 0{\cdot}05$ and eluted together as a single protein peak by 0·2M-NaCl.

Solutions made from preparations of rabbit LS stored for some time at 4° or −20° C. or stored solutions of LS preparations usually gave one line of precipitation when tested against anti-vaccinia sera. The reason for this behaviour is not understood. It was not due to a qualitative loss of one serological specificity since, as shown in Pl. 1, fig. 4, results similar to those shown in Pl. 1, fig. 1, could still be obtained with such solutions save that the LS–AVS reaction appeared to show only one line of precipitation which exhibited 'spur-formation' at the junction of the LS(H)–AVS reaction. The same figure shows, however, that the one line of precipitation formed in the LS–AVS reaction exhibited spur-formation with the reaction observed between a 'semi-specific' anti-L serum and the LS preparation under test. The inability of the 'semi-specific' anti-L serum to react with sheep S(PR) is shown clearly in Pl. 1, fig. 5. These results could have been obtained if the stored solution of rabbit LS used for test contained some degraded material which was L-inactive, S-active. Such a material could have diffused through the LS-('anti-L') precipitate and reacted with anti-S antibody diffusing from the well containing anti-vaccinia serum.

In some experiments more than two lines of precipitation were detected when these preparations of rabbit LS were tested at 10 mg./ml. against an anti-vaccinia serum. The preparations of rabbit LS which gave two bands in polyacrylamide gel electrophoresis were invariably heterogeneous when tested in this way even when examined at 1 mg./ml. Plate 1, fig. 6, shows that by study at a number of concentrations up to five lines of precipitation could be produced by the heterogeneous preparation LS 7. However, by suitable adjustment of the concentration of reagents the result shown in Pl. 2, fig. 7, was obtained. Comparison of the result with theoretical diagrams for preparations containing either discrete L and S substances or a single LS substance (Pl. 2, fig. 8*a*, *b*) suggests that a single LS substance was present in LS 7. Similar results were obtained with four of the five patently heterogeneous rabbit LS preparations. The atypical preparation behaved as though it contained discrete L and S substances.

Attempts were made to fractionate the heterogeneous rabbit LS preparations on DEAE-Sephadex (Pharmacia Ltd., Uppsala, Sweden). These were unsuccessful as useful resolution of components was not achieved.

Serological examination of the two materials obtained from vaccinia-infected sheep dermis showed that both substances were related to the LS material obtained from vaccinia-infected rabbit dermis. (Pl. 2, fig. 9). Plate 2, fig. 10, shows further that the serological activity of one material (sheep S) was stable to heating at 56° C. for 45 min. and that the serological activity of the other material (sheep L) was destroyed by such treatment. The figure shows also that the L and S substances isolated from vaccinia-infected sheep dermis were not related serologically to each other since the lines of precipitation formed in the S–AVS reaction and the L–AVS reaction cross each other. The discrete serological identity of L and S preparations isolated from vaccinia-infected sheep dermis is shown again in Pl. 2, fig. 11. Here also the lines of precipitation formed in an S–AVS reaction cross each other. Moreover in this figure the anti-S serum is shown to react with the S preparation and not the L preparation.

An attempt was made to relate rabbit LS to the poxvirus l.p.c. described by Rondle & Dumbell (1962). Plate 2, fig. 12, shows that an anti-cowpox serum (ACS) reacted with a rabbit LS preparation to give one sharp line of precipitation. The rabbit LS preparation used gave only one band in polyacrylamide gel electrophoresis. The line of precipitation formed was contiguous with one of the l.p.c. given by this serum when tested against vaccinia soluble antigen; it was not formed when cowpox soluble antigen replaced vaccinia. This l.p.c. was called 'f' by Rondle & Dumbell (1962). Further work showed that anti-cowpox sera varied markedly in anti-L and anti-S antibodies but that either antibody would give an 'f' line with vaccinia soluble antigen which could not be obtained using cowpox soluble antigen. This suggests that the classical L and S specificities are absent from cowpox soluble antigen as usually prepared although one specificity at least can be released from cowpox-infected rabbit dermis by treatment with trypsin (Rondle & Dumbell, 1962).

The L and S specificities of LS were found to correspond respectively to l.p.c. 3 and 5 of the standard vaccinia system described by Williamson (1963) and Baxby & Rondle (1968).

Quantitative serological studies

The smallest amounts of rabbit LS, sheep S, and sheep S(PR) that would give visible precipitates when tested against different anti-vaccinia sera were determined using gel-diffusion tests on microscope slides. Two materials, rabbit LS and sheep S(PR), were titrated at 1/1000, 1/2000, 1/3000 and so on; the other material, sheep S, was titrated at 1/50, 1/100, 1/150 and so on. The results are given in Table 1. It is evident that S(PR) was the most active material and hence the ratios of 'smallest reacting dose of LS or S/smallest reacting dose of S(PR)' were calculated for each antiserum and included in the Table. The agreement shown by the several ratios with the exception of S/S(PR) for serum 96 supports the contention that the same serologically reactive site (S) was titrated in each case. The ratios also show that the physically heterogeneous S(PR) was at least 20 times more active serologically than the physically homogeneous S preparation. This result is considered more fully in the Discussion but it must be stated here that the serological activity of solutions of crude preparations of sheep S was increased three- to fourfold when they were heated for 3 min. at 100° C. at pH 8·0 followed by the same heat-treatment at pH 6·0.

Table 1. *Quantitative serological reactivity of LS, S(PR) and S*

	Reciprocal of end-point titre			Ratio of titres	
Serum	LS	S(PR)	S	LS/S(PR)	S/S(PR)
93	10,000	16,000	7,500	1·6	21·4
96	12,000	21,000	6,000	1·7	35·0
97	10,000	16,000	7,500	1·6	21·4
99	10,000	16,000	7,500	1·6	21·4
104	10,000	14,000	6,000	1·4	23·4

DISCUSSION

The results given in this paper show that the preparation of LS antigen by classical methods from vaccinia-infected rabbit dermis yielded usually a heterogeneous material containing three or more virus-specific serologically reactive components. This is in agreement with recent work by Marquardt, Holm & Lycke (1965*a*) and Cohen & Wilcox (1966). For reasons not understood, occasional preparations were less complex both physically and serologically.

The isolation and study of the less complex materials followed by careful serological examination of the more heterogeneous substances supported the view of Craigie & Wishart (1936*a*, *b*) that extracts of vaccinia-infected rabbit dermis contain *inter alia* a single substance (LS) which possesses both a heat-labile (L) and a heat-stable (S) serologically reactive site. Under some conditions in the present experiments the supposed single substance gave two lines of precipitation when tested against suitable antisera in gel-diffusion tests. The two lines of precipitation were not due to separate L and S substances contaminating the LS material since treatment of the material with a specific anti-S serum abolished both the L and S reactions. That a single substance could give more than one line of precipitation when tested by gel-diffusion techniques has been demonstrated or postulated previously by Jennings (1954), Wilson & Pringle (1954, 1955), Kaminski (1955) and McDuffie, Kabat, Allen & Williams (1958).

Craigie & Wishart (1938) suggested that LS antigen from vaccinia-infected rabbit dermis might, under some conditions, dissociate into discrete L and S substances. This may have occurred with one of the seven preparations studied. Alternatively the earlier view could have been confused by the presence of the additional but then unsuspected antigens now shown to occur frequently in 'LS' preparations. Results with the other six materials suggested that stored preparations of LS or stored solutions of LS decomposed and behaved serologically as a mixture of LS and L inactive, S active substances. This finding is in accord with the views of Smadel & Shedlovsky (1942). Clear evidence for an LS antigen was obtained only from freshly prepared fractions of virus-infected tissue and such a substance was not very stable.

Despite repeated trial it was not possible to isolate a single LS substance from extracts of vaccinia-infected sheep dermis. It was possible, however, to use the technique described by Parker & Rivers (1937) and obtain a material S(PR) which was heat-stable and serologically indistinguishable from the S specificity of LS. It was also possible to fractionate the sheep material on DEAE-cellulose. This fractionation yielded two chemically and physically different substances, one possessing the serological specificity of L, the other that of S. No serological relationship could be demonstrated between the two materials; indeed, experimental results indicated that they were serologically distinct. The isolation from vaccinia-infected tissue of a material exhibiting L activity only has not been reported previously, although Smadel, Hoagland & Shedlovsky (1943) claimed to convert a purified LS preparation to an L active, S inactive substance by treatment with chymotrypsin. Since the sheep L preparation was shown to be physically heterogeneous, chemical studies of the material were not attempted and no comment can be made on the possibility that the sheep L substance could have arisen by degradation of a hypothetical LS substance present initially in extracts of vaccinia-infected sheep dermis. Comment can, however, be made in respect of the S substance isolated from vaccinia-infected sheep dermis by absorption–elution chromatography on DEAE-cellulose. This material was subjected to further treatments and isolated as a physically homogeneous substance of mol.wt. *ca.* 31,000 which contained *ca.* 75 % DNA (Williamson & Rondle, 1964). When tested against suitable antisera it gave an S reaction up to a dilution of 1 in 750. The LS preparations from vaccinia-infected rabbit dermis used for this paper contained less than 5 % nucleic acid but reacted as S containing materials up to dilutions of 1/10,000 when tested against those anti-vaccinia sera used for the titration of the sheep S preparation. Smadel & Shedlovsky (1942) quoted their purified preparation of rabbit LS as mol.wt. 250,000. The mol.wts., nucleic acid contents and serological activities of sheep S and rabbit LS are not compatible with the view that the homogeneous sheep S might have arisen by degradation of a hypothetical LS substance present initially in vaccinia-infected sheep extracts and similar in chemical composition to the LS present in vaccinia-infected rabbit dermis. It would appear that although the serological specificities L and S both occur in extracts of vaccinia-infected rabbit and sheep dermis, these specificities are not necessarily associated with identical 'carrier' molecules. It may be speculated that in different host tissues vaccinia virus is not synthesized by precisely the same biochemical pathways. At least these findings suggest that caution should be exercised when comparing results obtained on the synthesis of any one virus in different host tissues.

The final point of discussion is the quantitative difference in serological reactivity between sheep S(PR) and sheep S. The physically heterogeneous S(PR) was prepared by vigorous techniques. It contained little nucleic acid and was at least 20 times as active serologically as the physically homogeneous S substance prepared by much milder procedures. If it is assumed that the 75 % DNA in S was serologically inactive then it follows that the 'serologically active weight' of S was only one quarter of its actual weight. Since S(PR) contained not more than 6 % nucleic acid the serologically active weight of this substance represented not less than 94 % of the actual weight. Comparison of serologically active weights shows that in this respect S(PR) was only 5 or 6 times more serologically active than S. It is tempting to speculate that a difference in serological activity of this magnitude might have been due to a steric hindrance effect of the DNA moiety upon the serologically active moiety of S. The speculation is supported by the observation that vigorous heat-treatment of S resulted in an increase in serological activity. The heat-treatment might have degraded the nucleic acid and made available more serologically reactive material on S. The result also suggests that S(PR) might have been a degraded form of S.

SUMMARY

A material (LS) analogous to that described by Craigie & Wishart (1936*a*) has been detected in extracts of vaccinia-infected rabbit dermis. Gel-diffusion tests on this material support the view that it is a single substance possessing two distinct serologically reactive sites. The tests also show that under some conditions rabbit LS may give two lines of precipitation against suitable antisera and that this is not due usually to the dissociation of LS into discrete L and S substances.

A study of vaccinia-infected sheep dermis failed to give evidence of a single LS substance. Instead two chemically different substances were isolated, one having the serological specificity of L, the other that of S. The physical, chemical and quantitative serological properties of sheep S were such that it could not have arisen by degradation of an LS material similar to that found in vaccinia-infected rabbit dermis. On this evidence it is suggested that vaccinia virus is not necessarily synthesized in the same way in different host tissues.

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EXPLANATION OF PLATES

The terms LS, L, S, and S(PR) refer to those antigens or haptens so designated in the text; LS heated at 56° C. for 45 min. is represented as LS(H). Anti-vaccinia sera are represented as AVS, the specific anti-S serum as aS, and the anti-vaccinia serum mixed with S(PR) to abolish anti S activity as aL.

Plate 1

Fig. 1. The LS-AVS reaction shows two lines of precipitation. One of the lines of precipitation is contiguous with both the line present in the LS(H)–AVS reaction and the line present in the S(PR)–AVS reaction. The other line of precipitation is absent from these reactions.

Fig. 2. Dilution of LS with an equal quantity of preimmunization serum (LS/2) did not affect the reaction with AVS and two lines of precipitation were formed. All serological reactivity of LS was abolished, however, on admixture with an equal volume of aS (LS/aS).

Fig. 3. Soluble antigen from vaccinia-infected sheep dermis (V) gave a complex line pattern when tested against AVS. It gave only one line of precipitation when tested against aS.

Fig. 4. A stored solution of LS gave only one line of precipitation when tested against AVS. This line however shows spur formation at the junction of both the LS(H)–AVS reaction and the LS–aL reaction. This result which suggests that stored LS contains some free S is discussed in the text.

Fig. 5. Although S(PR) gives one line of precipitation when tested against AVS it fails to react with aL.

Fig. 6. The result obtained when the heterologous preparation LS 7 was tested against AVS depended upon the concentration of LS 7 used. LS 7 was at an initial concentration of 5 mg./ml. Dilutions of 1/2, 1/4, etc., were made with saline.

Plate 2

Fig. 7. By suitable selection of the concentration of LS 7 (here 0·8 mg./ml.) and by using LS(H) and specific aS results were obtained which suggested that LS 7 contained a single LS substance.

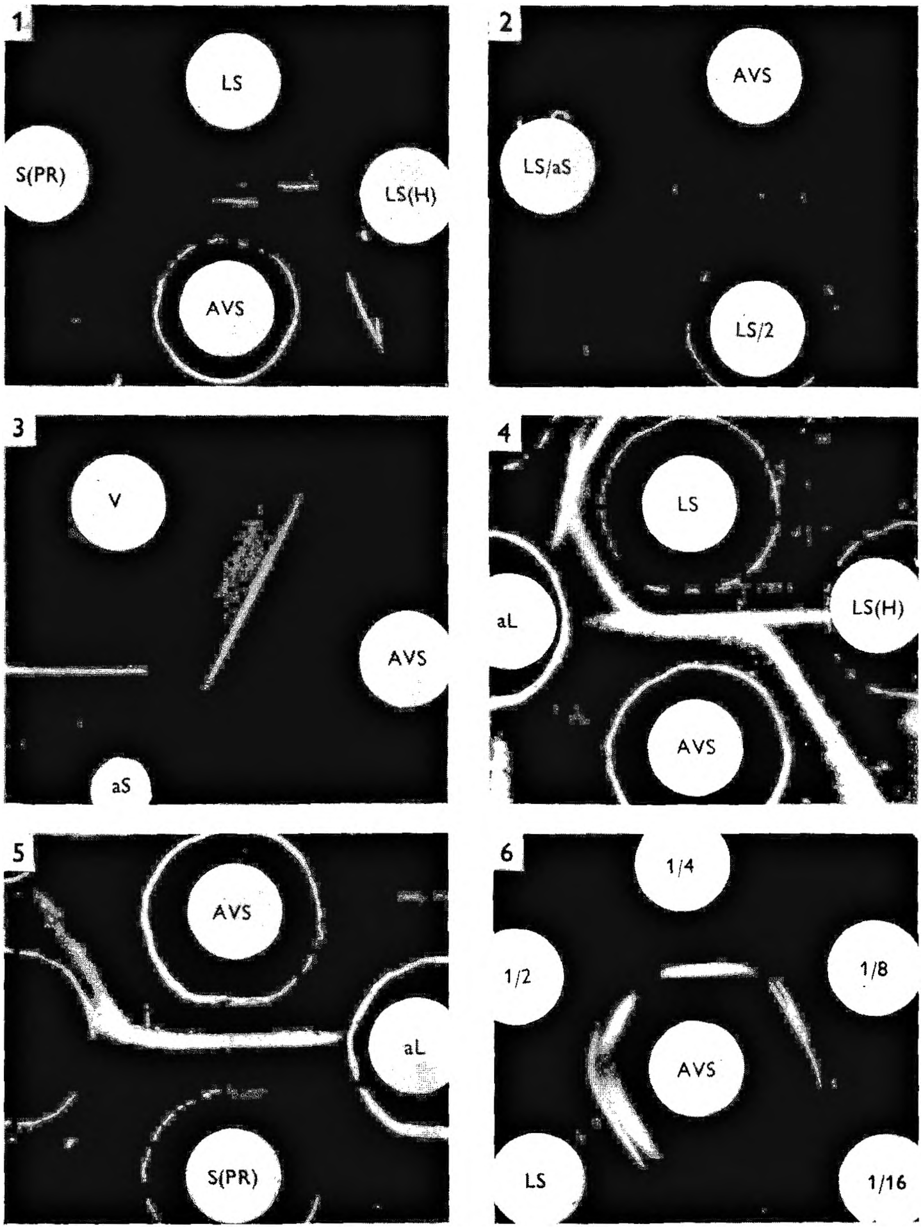
Fig. 8. (*a*) Theoretical result if no LS present, but L and S substances existed independently. (*b*) Theoretical result expected in the experiment shown in Fig. 7 if *inter alia* a single LS substance were present in LS 7.

Fig. 9. The L preparation obtained from vaccinia-infected sheep dermis is serologically identical with part of the LS–AVS reaction. The S preparation is also related to the LS–AVS reaction. As expected from the result shown in Plate 1, fig. 1, the S preparation is serologically identical with S(PR).

Fig. 10. This result shows that the S preparation was serologically stable to heating for 45 min. at 56° C. (HS). The L preparation lost its serological activity on conversion to HL by this heat-treatment. The crossing of the lines of precipitate formed in the L–AVS and the S–AVS reactions shows that the L and S preparations are serologically distinct.

Fig. 11. The specific antiserum aS reacts with sheep S but not sheep L. Both S and L, however, react with AVS to give single lines of precipitation. These single lines cross each other and indicate again that the L and S preparations are serologically distinct.

Fig. 12. Anticowpox serum (ACS) reacted with LS to give one l.p.c. This l.p.c. was given by soluble antigen from vaccinia-infected rabbit dermis (V) but not by soluble antigen from cowpox-infected rabbit dermis (CR).


1
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LS(H)
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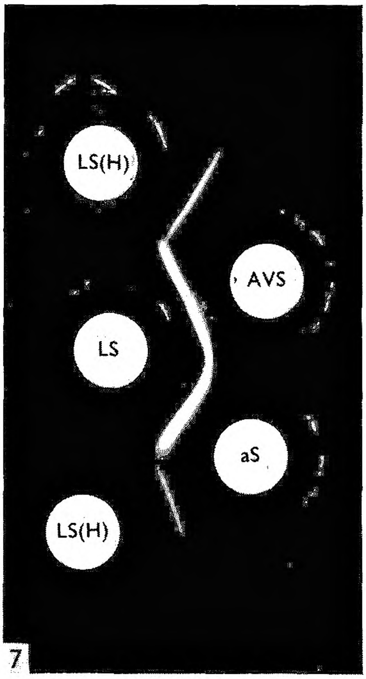
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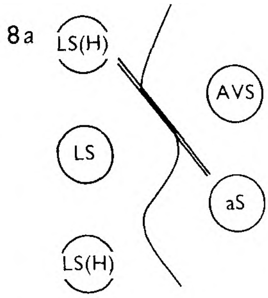
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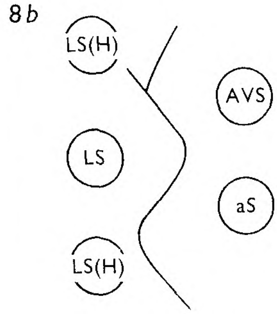
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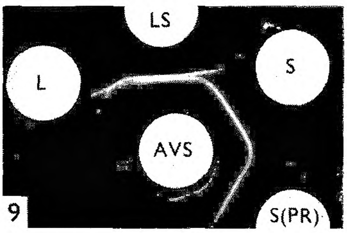
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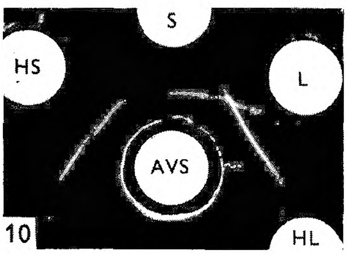
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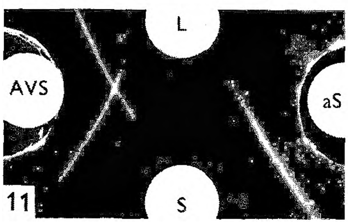
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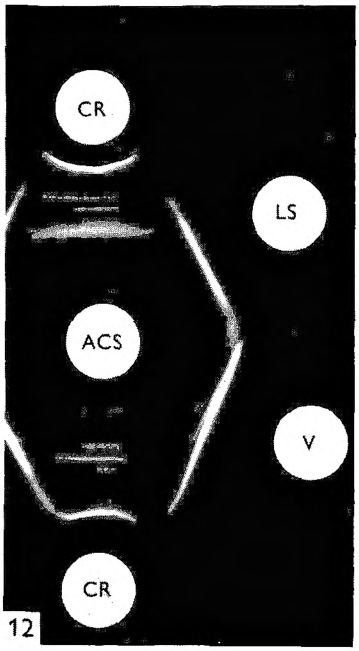
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The epidemiology of respiratory infection in an isolated Antarctic community

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INTRODUCTION

During 1965 and early 1966 a study in virus epidemiology was undertaken at Mawson, an Australian National Antarctic Research Expedition station. The aim of the study was to assess the incidence and aetiology of infection, particularly respiratory infection, in the station personnel.

Such a captive group, which has limited and known contacts, would seem to provide an excellent opportunity for an epidemiological study. Paul & Freese (1933) demonstrated the feasibility of conducting programmes in polar bacteriology, but virology under these conditions is more difficult. Because of the isolation and relatively small size of most establishments, virus culture is not possible, though specimens can be collected for later culture attempts. This is satisfactory for some of the more stable virus groups but infection with other viruses can be confirmed only by detecting changes in the antibody titre of serial serum samples.

Two virology programmes had been attempted in Antarctica before 1965. Sladen & Goldsmith (1960) were engaged on 'Operation Snuffles' during the summer of 1958–9. This included studies on fifty-six volunteers from the 232 men on the icebreaker U.S.S. *Staten Island*, who gave monthly blood specimens for antibody assay. Each man had a total of fifteen throat swabs taken for virus culture before entering and after leaving ports of call on the voyage and whilst in Antarctica. Less than 50% of the total ship's complement complained of upper respiratory tract symptoms after leaving these ports of call. In addition, serum specimens were collected from the entire complement of the U.S.S. *Staten Island* at the beginning of the voyage and before returning to America, a period of approximately 6 months. Blood and throat swabs were also collected from personnel at two Antarctic stations; Hallett which was semi-isolated and Wilkes which had been completely isolated for 1 year. The Wilkes party reported some minor symptoms after contact with M.V. *Magga Dan*, which had arrived a few days before U.S.S. *Staten Island*. Neither party had epidemics of colds or sore throats after boarding the U.S.S. *Staten Island*. A final unpublished phase of this study was the collection of similar specimens from wintering parties at McMurdo Sound. All specimens were sent to Dr R. M. Chanock, who tested the sera for the presence of antibodies to a number of respiratory viruses, not including rhinoviruses. No evidence of infection was shown with the viral antigens tested (R. M. Chanock, personal communication). Following this a systematic study of serum from members of the South African National Antarctic Expeditions was carried out during 1961 and 1962 to detect virus antibody in monthly serum specimens. This survey did not show any evidence of new virus infection during this expedition (J. H. S. Gear, personal communication).

METHODS AND MATERIALS

Logistics

Mawson (latitude 67° 36′ S, longitude 62° 53′ E) is approximately 3600 miles and 10 days' sailing from Melbourne. Budd (1964) fully discusses the living conditions and the type of work performed at this Station. The party under study arrived at the station early in January, 1965. Their ship left the Mawson area with the 1964 party aboard 4 weeks later. The twenty-seven new personnel remained isolated from then until January 1966, when there was a brief visit by a small party of Russian airmen who had already spent more than 1 month in Antarctica. The final relief took place in February and the 1965 party arrived back in Hobart in March 1966. The relief party had been at sea and at Wilkes for approximately 1 month before reaching Mawson.

Clinical data and specimen collection

The medical history of each subject was recorded from December 1964 until March 1966. Further inquiries were made by questionnaire in June 1966. Throat swabs were taken and kept in 2 ml. of virus transport medium at $-70°$ C. (Equal parts of Hanks's balanced salt solution and Parker 199 medium with penicillin 100 units, streptomycin 100 μg., neomycin 20 μg. and amphotericin B 5 μg. in each bottle.) Nose swabs were collected with cotton-tipped applicator sticks moistened with the same medium and were also stored at $-70°$ C. Specimens of faeces 5–10 g. in weight were collected by the men and placed in sterile 1 oz. screw-capped bottles for storage at $-70°$ C. Serum preparation from clotted blood samples proved unsatisfactory because of unexplained haemolysis in many of the specimens; consequently, plasma was prepared from citrated blood. This proved suitable for antibody assay and gave similar results to serum samples collected from the same subject. Specimens were stored in mechanical refrigerators until the virological tests at the Institute of Medical and Veterinary Science (I.M.V.S.) were completed. Blood smears were prepared from all blood specimens and leucocyte counts were performed on those samples collected at Mawson.

Virus isolation procedures

Virus isolation was attempted using a range of cell-culture systems at temperatures and hydrogen ion concentrations considered optimal for the growth of virus groups implicated in upper respiratory tract infection. The cell systems are detailed below:

(i) Primary monkey kidney (PMK) cell cultures were supplied as cell suspensions from *Maccacus cynomolgous* monkeys by Commonwealth Serum Laboratories (C.S.L.), Melbourne.

(ii) The HEp-2 cell line (Toolan, 1954) used in this study was originally received from C.S.L. and has been maintained at the I.M.V.S. for a number of years.

(iii) Two strains of human foetal diploid cells were used. The first was a foetal lung strain (HFDL) supplied by C.S.L. and had been shown to support the growth of some rhinoviruses. The second was the WI-38 strain kindly supplied by Dr L. Hayflick of the Wistar Institute, Philadelphia. The culture methods used for these strains were those described by L. Hayflick (personal communication).

The maintenance media for the cell monolayers are outlined in Table 1. In addition, all media contained 2 % foetal calf serum, 100 units of penicillin/ml. and 100 μg. streptomycin/ml. Leibovitz medium number 15 was used to maintain cultures inoculated with specimens from the final four collections of the study and all of the human foetal diploid cultures.

Table 1. *Composition of maintenance media used for cell culture and incubation temperatures used in this study*

Inoculum	Cell culture and incubation temperature		Formula*	Sodium bicarbonate (ml. of 4·4 % solution)	Sodium hydroxide (ml. of N solution)
Nose and throat	HEp-2	37° C.	Eagle's MEM in Earle's BSS	2·8	—
	HEp-2	37° C.	Leibovitz 15	—	0·15
	PMK	33° C.	Eagle's MEM in Earle's BSS	1·9	—
	PMK	33° C.	Leibovitz 15	—	—
	HFDL and WI-38	33° C.	Leibovitz 15	—	—
Faeces	PMK	37° C.	Eagle's MEM in Earle's BSS	2·8	—
	PMK	37° C.	Leibovitz 15	—	0·15

* GIBCO dehydrated media used throughout, see Grand Island Biological Co., New York, *Price and Reference Manual*, 1966.

Because of the large number of nose and throat swabs collected in this survey, the complementary samples were pooled in the laboratory and processed as one specimen in HEp-2, PMK, HFDL and WI-38 cell cultures which were subsequently scanned for cytopathic changes. Specimens were maintained in HEp-2 cell cultures for 28 days which entailed passaging the culture, after three cycles of rapid freezing and thawing at 14 days. The PMK cell cultures were tested for haemadsorption (Chanock & Johnston, 1964) 14 days after inoculation to detect the presence of myxoviruses. Haemadsorption tests were similarly performed on the human foetal diploid cell strains 21 days after inoculation.

The specimens of faeces were suspended in Eagle's medium plus 2 % foetal calf serum and inoculated into PMK cell cultures and maintained for 21 days. When toxicity resulted in early cell degeneration a further passage was performed.

Serology

The standardized straight-line technique of complement fixation test was employed to detect antibodies against influenza A and B, mumps, adeno-virus, Herpes simplex and ornithosis. The method has been described by Bradstreet & Taylor (1962).

RESULTS

Clinical data

There were thirteen men who complained of symptoms of upper respiratory tract infection within 2 weeks of leaving Melbourne. These cases were mild while on the ship, but several of these men developed a cough once they began working at the station. The clinical course of these colds ranged from 1 to 2 weeks and were the only cases recorded until January 1966. No infective diseases were observed during the period of isolation. It was not uncommon, however, to have a sore, dry throat on waking in the morning, presumably a result of breathing the warm, dry air of the sleeping huts. This discomfort would disappear after a few hours. This phenomenon was noted at other Antarctic stations (K. E. Hicks, personal communication) and has also been observed in the Canadian Arctic (Schmidt, 1963).

The Mawson geophysicist (J.E.H.) worked with a Russian field party from 3–9 January 1966, during which time he had some rhinorrhoea and transient dry throat. He returned to Mawson and suffered from mild abdominal pain and loose, but not more frequent, bowel motions from 11 to 13 January. Ten days later many of the party had diarrhoea and some abdominal colic, apparently related to the eating of mutton that had been frozen and thawed several times. During the afternoon and evening of 26 January four men, including J.E.H., were engaged in hosing down the station area with seawater pumped from beneath the harbour ice. While manœuvring the canvas hose their hands and patches of clothing not covered by rubber over-garments became damp and cold, but subjectively they did not feel any greater cold discomfort than they had experienced during the previous year. Thirty-six hours later, three (including J.E.H.) of these four men noted the onset of sore throat, mild rhinorrhoea and muscle aches. The sore throats persisted for 1 week.

The relief ship arrived on 3 February and during the changeover and on the return voyage six other men had similar respiratory symptoms, but all the cases were mild. No fever was noted, clinical examination of the fauces and pharynx showed nothing abnormal, and cough when present was irritative and not productive.

By questionnaire it was found that seventeen men, including five who had infections during the relief and on the return voyage, developed upper respiratory tract infection of moderate severity on returning to Australia. Twelve of them developed symptoms within a fortnight of disembarkation, and symptoms lasted from 7 to 14 days.

Virus isolation and serology

All attempts at virus isolation using the systems detailed above from the throat, nose and faeces specimens were unrewarding. There were no diagnostic rises in antibody titre to any of the antigens tested. The scattering of the men on their return unfortunately precluded the collection of further specimens.

Leucocyte studies

The total leucocyte counts done through the year remained relatively constant for each man, as did the differential white blood cell percentages. In some instances, marked increases in the ratio of polymorphonuclear leucocytes to lymphocytes occurred during the voyages but they correlated poorly with symptoms. Total white blood counts could not be performed while at sea so the significance of these changes is unknown.

DISCUSSION

The clinical findings in the 1965 Mawson party were consistent with the observations made during previous and subsequent expeditions. The 'burning out' of respiratory infection (Tyrrell, 1965) began when the party, drawn from all over Australia, sailed south in a small ship, living four or five to a cabin. This wave of infection continued for a week or so after arrival at the station, during which time the men spent long hours in strenuous outdoor activity in temperatures around freezing point and with wind speeds up to 60 knots. It is understandable that the men who landed with colds should have had an exacerbation of symptoms at this time. Seven of the 1966 Mawson party similarly had upper respiratory symptoms during their voyage to Wilkes (J. Hudson, personal communication) and many of the 1966 Macquarie Island party developed moderate to severe colds once they had reached the Island (D. Edwards, personal communication). Two of the latter party were confined to bed because of an accompanying fever. Taylor (1960) and Hedblom (1961) also noted moderately severe upper respiratory tract infections in newcomers to American stations in Antarctica.

Once established at Mawson, the 1965 party did not report any more colds for almost a year. This observation is in keeping with the experience of other expeditions on which adequate records have been kept (Wilson, 1965). This phenomenon appears to be a function of the size of the community and its isolation. A population of about 500, as in the Spitzbergen study by Paul & Freeze (1933), is near to a critical size for the perpetuation of colds throughout the year. Most Antarctic populations do not number over thirty during the winter, thus there are a limited number of subjects susceptible to any virus pool left in such a group after the first few weeks. Though it is conceivable that some virus groups capable of prolonged colonization of various organs (such as adenoviruses and enteroviruses) may remain, the men's physical isolation ensures that new viruses will not be introduced.

Much interest has centred around the epidemics of respiratory tract infections that have been seen to devastate some isolated communities when they are contacted by outsiders (Andrewes, 1965). We believe a distinction should be drawn, however, between naturally isolated peoples and the personnel of modern polar expeditions. Examples of the former group include the Eskimos with their history of explosive outbreaks of infectious diseases such as poliomyelitis (Reinhard & Gibson, 1960) and influenza (Reinhard, 1962; Philip & Lackman, 1962). Heyerdahl (1958) also describes a wave of respiratory infection known locally as a 'coconga' which affects the Easter Islanders following visits by a ship. Similar outbreaks of respiratory tract infection afflicted the islanders of Tristan da Cunha when visited by ships which had recently left larger centres of civilisation (Woolley, 1963). There are even groups of aboriginals in Australia at present who have had little contact with outsiders, and with their relatively inexperienced immunological status, can be decimated with a viral disease such as measles (Tooth & Lewis, 1963). Virus diseases in this type of community with its limited experience of infection have high morbidity and mortality rates. This susceptibility contrasts with that seen among personnel on polar expeditions even though they are isolated for a period of 12 months or more. There are a number of relevant observations that have been made in Antarctica in the last 10 years. Taylor (1960), the Medical Officer at McMurdo Sound during 1956, records that on the relief of his party, some colds were experienced, but were mild in comparison with those suffered by the relieving party. Siple (1960) spent the winter of 1957 at the South Pole Station and records an unusually severe epidemic of upper and lower respiratory tract infection both in his party and in the crew of the aircraft which visited them in late October. It would appear, though, that this was a special situation and that the airmen were actually infected with Asian influenza, which was epidemic at the time. This would explain a high rate of infection in the wintering-over party and the severity of the symptoms. This specific instance cannot be taken, then, as the typical reaction of a modern Antarctic community on breaking isolation. Records were kept at McMurdo Sound of the number of upper respiratory tract infections amongst the 1959 wintering-over party and the incoming relief personnel (Hedblom, 1961). Again, one notes the relatively few infections suffered by the wintering-over party whilst still in Antarctica: 16 cases among approximately 80 men, as compared with 134 cases among approximately 170 of the newcomers.

This final phase of the period of isolation for the 1965 Mawson personnel was complicated by the contact with the Russian aircrew, which lasted approximately 12 hr., and by the presence of one member of the party (J.E.H.) who had been with a larger Russian party for 6 days. His description of his upper respiratory symptoms during this time was suggestive but not conclusive of infection, and his abdominal symptoms on returning to Mawson were of uncertain origin and significance. More definite, however, is the fact that three men, including J.E.H., developed some upper respiratory tract symptoms 18 days after the Russian contact and within 36 hr. of an episode of chilling which lasted from 3 to 4 hr. It can be postulated that a virus was seeded into the Mawson population directly by the visitors or indirectly by J. E. H. and that the later apparent infections were examples of virus activation (Andrewes, 1965) in men who were engaged in a cold job for some hours. There was no evidence of cross-infection from these men to the other personnel. Another factor which may have played a part was a general climatic change with a significant temperature drop (Holland, Spicer & Wilson, 1961; Sutton, 1963) which occurred at this time. This would suggest that if virus activation is a reality, it is dependent on a number of environmental factors, and may explain the difficulty experienced in inducing activation of infection in volunteer experiments.

Three of the second series of infections were reported 3–5 days following the arrival of the relief ship and the rest were at weekly intervals after that. This series of cases as observed by one of us (A.S.C) fell into the doubtful mild cold and mild cold classes described by Tyrrell (1965) and can be taken as evidence of infection (Roden, 1958). Resistance to infection and modification of an infection is related to the possession of protective antibody against the challenging virus. It was interesting to note that the low levels of complement-fixing antibody present at the beginning of the expedition did not drop significantly during the year of isolation. McLean (1919), Adams & Stanmeyer (1960) and Sladen (1965) report that the bacterial flora of the mouth and pharynx decreases during a year in Antarctica. Ritchie (1958) has suggested that colds are prolonged by the secondary bacterial infection of virus-damaged tissues. It is tempting to suggest that the mildness of some of the colds experienced by men who have wintered in Antarctica could be explained by the relatively low-grade secondary infections by their depleted pharyngeal flora. There was, however, no apparent diminution of pharyngeal flora seen in this Mawson group in a separate bacteriological study, though quantitative methods were not used.

These observations suggest that there is a reduced susceptibility to colds in many wintering-over parties while still on the ice, though exceptions have been recorded. Wilson (1965) quotes an experience recounted by Dr Goldsmith concerning members of a wintering party from Halley Bay, many of whom caught severe colds on boarding their relief ship. Several days later, however, these men, still suffering from their colds, came into contact with another wintering party from Shackelton Base, none of whom caught colds. A similar situation was noted at Macquarie Island when the 1965 party there failed to contract respiratory infection despite the presence of the currently infected 1966 relief expedition (D. Edwards, personal communication). It is possible that the relief parties challenged the respective wintering-over parties with viruses to which the latter were immune. This, however, must be a moderately uncommon situation in view of the multiplicity of rhinovirus serotypes now recognized. The work of Hemmes, Winkler & Kool (1960) and Buckland & Tyrrell (1962) may have a bearing on virus survival and spread under Antarctic conditions where the relative humidity in the heated living huts remains from 15 % to 30 %, favouring the survival of influenza, parainfluenza and respiratory syncytial virus, but limiting the effective transmission of other virus groups. If this is a factor, one may expect an inverse effect at a sub-Antarctic station such as Macquarie Island where a constant high humidity prevails, possibly encouraging the survival of the ether-stable viruses such as the adenovirus group and the picornaviruses.

The final phase of the present study began when the men reached Australia. More than half of the group had definite upper respiratory tract infections within 2 weeks of disembarkation. Subjective assessment only is available for these cases, but save for one or two, these infections were not more debilitating than the colds usually experienced by the subjects. This is a significant finding and it is interesting to note that there were many more definite infections at this time than were noted in Antarctica on breaking isolation and that the infections suffered in Antarctica conferred no significant protection against contracting infection on return to Australia. Though this type of information has not been obtained previously, it is of great significance in this type of study and we suggest that further surveys be conducted. As it is almost impossible to do it by personal interrogation and examination, questionnaire appears to be the only method of obtaining the data.

In summary, the sequence of events during an Antarctic expedition may be tabulated as follows:

(i) The party assembles and boards a ship. This crowding enables spread of infection to occur if there are virus excreters in the group, as colds are usually experienced on the voyage.

(ii) These colds may burn out if the voyage is long (over 1 month); but usually, on arrival in Antarctica and with much greater exposure to cold, infections continue to appear. Activation of latent infections is conceivable during this period and there is symptomatic worsening of existing colds.

(iii) When this wave of infection has ceased, upper respiratory tract infections are absent for the remainder of the period of isolation.

(iv) Mixing with the relief expedition usually results in some cases of respiratory infection in the wintering-over party while still in Antarctic regions, but these infections are notably mild and spread of infection is apparently not highly effective.

(v) Within a short period of returning to normal community life, colds of moderate severity, involving the majority of the personnel, can be expected. In some situations as yet undefined, these infections may occur while still in Antarctica.

Unfortunately, the several attempts to define the aetiological agents responsible for colds in Antarctica by serological and isolation studies have been unsuccessful. In spite of this, these Antarctic groups with their months of freedom from upper respiratory tract infection do provide excellent opportunities for basic epidemiological study. Surveys similar to those reported above should be continued, laying stress on a complete clinical coverage before, during and after the Antarctic sojourn. Careful specimen collection should be attempted during the relief period to try to establish which virus groups can cause colds under Antarctic and sub-Antarctic conditions. We believe that experimental inoculation of volunteers in these groups, using easily traced viruses, would yield valuable results. The general moral and ethical issues involved in the conduct of volunteer experiments have been discussed by Jackson *et al.* (1963) and their conclusions remain valid for these isolated groups in view of the fact that the clinical manifestations of infection by most common cold viruses are mild.

One does not find difficulty in obtaining volunteers on these expeditions to take part in unpleasant procedures if the reasons are adequately explained and they feel that their participation is appreciated and will provide useful information. These groups would be ideal for the studies in the fields of virus persistence, antibody response to infection and resistance to serial reinfection by the same or different virus strain. Transmission experiments could also be attempted, for at a station such as Mawson there are numerous buildings which could be used, each with a thermostatically controlled heating system and stable relative humidity. Because there are trained weather observers at these stations, accurate recording of macro- and micro-meterological data does not present a problem. Medical laboratory facilities are adequate to allow simple haematological data such as white blood cell counts and erythrocyte sedimentation rate to be collected as described by Cate, Couch & Johnson (1964) for rhinovirus infections, before, during and after experiments.

SUMMARY

The results of a combined clinical and laboratory study of respiratory infections among members of an Australian Antarctic expedition are presented. Virus isolation and serological methods were employed, but the aetiological agent or agents responsible for respiratory infections in this group were not revealed.

The clinical findings were correlated with published and unpublished studies on comparable communities, and the following broad pattern of the epidemiology of respiratory tract infections in such groups has emerged. The assembly on board ship of a party from widely separated areas often leads to infections after embarkation. On long voyages these infections may burn out, but frequently new cases are still appearing when the party arrives in Antarctica. Symptoms in sufferers at this time become more severe, suggesting that the sudden environmental change from the warmth of an air-conditioned ship to the harsher Antarctic conditions may influence the course of the respiratory infections. With isolation established in Antarctica, further cases rarely appear. On exposure to the relief party, however, infections can be expected, and it is noted that morbidity is usually low and symptoms are mild, indicating an apparent heightened resistance to infection in the acclimatized party while still on the ice.

This study has further shown that most of these men, on returning to urban societies, contracted moderately severe upper respiratory tract infections in contrast to their apparent resistance when under Antarctic conditions.

The suitability of these groups for experimental study, including the inoculation of volunteers, was discussed and suggestions were made for future work.

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The hygiene of slicing machines, carving knives and can-openers

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The work described in this paper was the outcome of requests to the Food Hygiene Laboratory for information concerning the effective cleaning of machines for slicing cooked meat in shops and cafés. It appears from the literature that data are not available although many bactericidal detergents and 'detergent/sterilizers' are advertised as being suitable cleaning agents. Information on the effective cleaning of large commercial can-openers and carving knives used for cutting cooked meat is also required.

The choice of detergents and detergent/disinfectants in the food and catering industries is governed by three factors: the efficiency of removal of a wide variety of food debris (in this case the protein and fatty materials associated with cooked, open packs or canned meats), the bactericidal activity of the substance(s) in solution, and the effect of the solution on the hands of personnel and on machinery.

The purpose of the work was not to compare the merits of commercial detergents and detergent/disinfectants but to find out the bacterial counts associated with slicing machines, can-openers and carving knives used for cooked or canned meats, to suggest a simple and effective method of cleaning these articles and to outline the methods available for testing the efficiency of cleaning.

The design and safety aspects of equipment were also considered. Whilst we accept that it is impossible to maintain sterility of food-handling equipment, more positive advice and information could be included in the Food Hygiene Regulations regarding methods for the cleaning of equipment and storage of cold meats. However, the Ministry of Health is at present preparing a code of practice entitled 'Hygiene in the Retail Meat Trade including Cold Cooked Meats and Meat Delicatessen'. This code of practice will include advice on the hygienic handling, storage and display of cold cooked meats, and on the cleaning of food slicing machines. A similar code of practice has been given by Hughes (1960).

MATERIALS AND METHODS

Bacterial counts from slicing machines, carving knives and can-openers before and after a simple cleaning procedure

An extensive survey was made at one modern large supermarket (Table 1, premises 1) by arrangement with the directors of the company and the manager.

The supermarket was clean and well organized and the staff had been instructed in the importance of hygienic food handling. Twelve visits were made at weekly intervals. On the first eight visits cleaning procedures were carried out by the staff usually responsible for this work. On the remaining four visits all cleaning procedures were carried out by one of the authors (R.J.G.). The technique and cleaning materials used were the same as before but the time taken to complete the prodecure (8–10 min.) was generally less than that required by the shop staff (9–13 min.). These times included the filling of buckets with hot water, the stripping, cleaning and re-assembly of one slicing machine, and the cleaning of one carving knife and one can-opener.

A limited survey was also made in two other towns at three supermarkets, three transport cafés, two grocery shops and in the food department of one chain store. All these visits were made with Public Health Inspectors; the managers of these premises were not warned about the visits. Details of all the premises and the frequency of cleaning the slicing machines are given in Table 1.

Table 1. *Details of premises examined and frequency of procedure used for cleaning slicing machines*

Premises	Business	Slicing machine manufacturer	Normal frequency of cleaning procedure
1	Supermarket	Asco-Bizerba	Midday and evening
2	Grocery shop	Crypto	Midday and evening
3	Grocery shop	Berkel	Midday and evening
4	Supermarket	Berkel	Evening
5	Supermarket	Berkel	Evening
6	Transport café	Zet Zimmerman	Evening
7	Transport café	Berkel	Evening
8	Transport café	Crypto	Weekly
9	Chain store (food dept.)	Berkel	Evening
10	Supermarket	Berkel	Evening

The size of slicing machines varied between premises: the total area of the two sides of the cutting blade was usually about 200 cm.^{2}. Duplicate segments of area 30 cm.^{2} from the top and bottom surfaces of the blade were swabbed using two calcium alginate swabs (Higgins, 1950) for each segment. The first swab was moistened in 9 ml. of quarter-strength Ringer's solution, contained in small screw-capped bottles, rubbed over the appropriate area and then broken off into the Ringer's solution; the second swab was used to remove excess moisture and any remaining food debris in the swabbed area, and broken off into the same Ringer's solution. The bottles of Ringer's solution containing the swabs were placed in vacuum flasks at 4° C. before carriage to the laboratory. On arrival, 1 ml. of a 10 % solution of sodium hexametaphosphate was added to each bottle, the solution was shaken gently for a few minutes to dissolve the swabs and the suspension was used for making plate counts. The same technique was used for swabbing carving knives and can-openers. Four swabs were used for one side of a carving knife blade; particular attention was paid to the area around the joint between handle and blade. The size and shape of carving knives varied between premises; the area swabbed measured 60–80 cm.^{2}. All the can-openers examined were mounted on working benches or tables. The areas swabbed were 1 cm.^{2} (blade) and about 4 cm.^{2} (area above the blade).

The effect on plate counts of using various fluids for swabbing and dilution was examined: tests were also made to ascertain the effect of storage at 4° C. in quarter-strength Ringer's solution on plate counts. All plate counts were made on blood agar using a modified Miles & Misra (1938) technique with incubation for 48 hr. at 22° and 37° C.

Most of the slicing machines examined in the present study were of modern design, yet some of the machines lacked satisfactory safety devices such as preventive guards; one was about 15 years old, most of the mechanical parts, including the cutting blade, were well worn and the machine appeared to be in need of a complete overhaul. The commercial can-openers examined were satisfactory for the job they were designed to do but they were difficult to clean efficiently. Scrubbing with a nylon or plastic brush to remove food debris on the mechanical parts was necessary before the application of a cleaning solution.

The staff at premises 1–10 used a wide variety of detergents or detergent/disinfectants. The cleaning done by one of the authors (R.J.G.) at premises 1, 9 and 10 employed the detergent/disinfectant regularly used at premises 1. No tests were made after cleaning at premises 2–8.

Cleaning procedure

Approximately 68 g. of the detergent/disinfectant powder, measured by filling a conical paper cup, was placed in a 2 gal. plastic bucket containing water at 48–50° C. The solution contained about 20–22 p.p.m. available chlorine and had a powerful detergent action. Cloths, disinfected overnight by immersion in a bucket of water containing 50–80 p.p.m. available chlorine, were used to clean the slicing machine, carving knife and can-opener with the prepared solution.

The cleaning technique used for slicing machines was as follows:

(1) The machine was switched off and the power plug removed.

(2) The cutting number was returned to zero.

(3) Carrier guards and trays were dismantled and the knife centre disk removed.

(4) Surplus food debris was removed from both sides of the blade with a plastic scraper.

(5) Both sides of the blade were cleaned by drawing a cloth, wrung out from the prepared solution, from the centre to the edge of the blade rotated by hand.

(6) The remainder of the machine was cleaned with the same solution.

(7) All parts were rinsed with hot water (50° C.) and allowed to dry.

(8) The machine was reassembled.

Laboratory tests with the cleaning solution

A simple minimum inhibitory concentration test (Kelsey, Beeby & Whitehouse, 1965) was carried out to discover if the solution would provide sufficient disinfectant activity to prevent the growth of a variety of organisms. Five cultures of coagulase-positive staphylococci, eight strains of salmonella (*S. typhi* NCTC 786 and NCTC 3390, *S. paratyphi B*, *S. typhimurium* phage-type 15a, *S. typhimurium* (untypable), *S. eimsbuettel*, *S. panama* and *S. worthington*) and one unidentified strain each of *Bacillus* sp. and *Micrococcus* sp. were used. With the exception of the two strains of *S. typhi*, all the cultures were isolated in the laboratory from food. For each organism two sets of 5 ml. doubling dilutions of the cleaning powder in nutrient broth were made. One set of the dilutions was kept at room temperature (22° C.) for inoculation and the second set was heated in a water bath to 45° C. and inoculated immediately after removal from the bath. An overnight broth culture of the organism to be tested was diluted 1/10 with nutrient broth and one drop (0·02 ml.) was used to inoculate each dilution in the two series. The inoculum contained approximately 200,000 organisms in one drop. After inoculation all the dilutions were incubated at 37° C. for 72 hr.

In-use test

On one occasion an in-use test (Kelsey & Maurer, 1966) was carried out at premises 1. After the slicing machine, can-opener and knife were cleaned, samples of the used cleaning solution were taken from the bucket which contained the cloth. Two 1 ml. samples of the solution were removed with a pipette and added to separate 9 ml. quantities of quarter-strength Ringer's solution. Two more 1 ml. samples were taken and added to separate 9 ml. quantities of inactivator broth (0·5 % sodium thiosulphate in nutrient broth). After returning to the laboratory, drops from each Ringer's solution and from each inactivator-broth were placed on duplicate agar plates. One of each pair of plates was incubated at 37° C. and the other at 22° C. for 48 hr.

RESULTS

Table 2 shows the effect of using various diluents for swabbing areas of a slicing machine after use, and after the given cleaning procedure by shop staff in premises 1; the same diluents were used for making tenfold dilutions before plating. The results show that plate counts were very similar with all diluents used. Since each area was swabbed with a different diluent it was assumed that the distribution of food and micro-organisms was fairly uniform around the whole circumference of the blade. This assumption was verified in subsequent experiments when duplicate pairs of swabbed areas from the top and bottom surfaces of slicing machines usually gave similar plate counts. In all subsequent work quarter-strength Ringer's solution was used as diluent.

Because of the delay between swabbing and dilution and plating, experiments were made to determine the effect of storage of samples in diluent at 4° C. In two experiments initial dilutions were stored at 4° C., and further dilutions were made at various time intervals before plating; in two other experiments all dilutions were made at once, before storage at 4° C. and plating. Table 3 shows that plate counts were unaffected by storage of initial dilutions at 4° C. for 5 hr.

Table 4 shows the range of plate counts from swabbed areas of slicing machines in various premises after normal use and after the given cleaning procedure. Each result represents the mean from duplicate swabbed areas. Visits to premises 1–5, 9 and 10 were made between 11.00 a.m.–noon and to premises 6–8 between 2–4 p.m. All the slicing machines examined had been in use for at least 2 hr. before swabbing and, in most cases, various types of cooked and canned meats had been sliced: the most popular products sold sliced were ham, pork luncheon meat, corned beef, jellied veal, ox tongue, liver sausage, savoury sausage, salami and roast pork.

Two temperatures of incubation were selected to represent normal room temperature (22° C.) and the optimum growth temperature for pathogenic bacteria

Table 2. *Bacterial plate counts at 22° and 37° C. from a slicing machine swabbed with various diluents, before and after cleaning*

		Total viable counts/swabbed area			
		Before cleaning		After cleaning	
Expt.	Diluent*	22° C.	37° C.	22° C.	37° C.
1	A	137,500	135,000	6,625	2,875
	B	275,000	225,000	5,500	2,750
	C	225,000	137,500	5,750	2,500
	D	125,000	67,500	3,500	3,000
2	A	375,000	387,500	33,750	22,115
	B	375,000	275,000	27,500	20,000
	C	350,000	275,000	20,000	12,500
	D	225,000	200,000	12,500	10,000

* A, Quarter-strength Ringer's solution; B, water + peptone (0·1 %); C, water + peptone (0·1 %) + Tween 80 (0·1 %); D, water + peptone (0·1 %) + Tween 80 (0·1 %) + sodium chloride (0·85 %).

Table 3. *Effect of storage at 4° C. in quarter-strength Ringer's solution on the bacterial plate counts at 22° and 37° C. from swabbed areas of a slicing machine*

		Total viable counts/swabbed area	
Experiment	Storage time (hr.)	22° C.	37° C.
1*	1	2,250,000	2,250,000
	2	2,250,000	1,625,000
	3½	3,000,000	2,000,000
	5	3,250,000	2,120,000
2*	1	4,250,000	6,000,000
	2	4,500,000	5,750,000
	3½	7,250,000	5,500,000
	5	8,250,000	7,000,000
3†	1	1,750,000	1,100,000
	2	925,000	825,000
	3½	825,000	350,000
	5	875,000	475,000
4†	1	450,000	375,000
	2	350,000	300,000
	3½	250,000	275,000
	5	375,000	275,000

* Initial dilution stored at 4° C.; further dilutions made before plating.
† All dilutions made at once and stored at 4° C. before plating.

(37° C.); counts were nearly always higher after incubation at 22° C. than at 37° C. As would be expected, the variations in plate counts were considerable. After incubation, plates were examined to determine the types of bacteria present. Before cleaning, micrococci, coliforms and aerobic sporing bacilli were usually found and, on several occasions, α-haemolytic and non-haemolytic streptococci,

Table 4. *Range of bacterial plate counts at 22° and 37° C. from swabbed areas of slicing machines before and after cleaning*

Premises	No. of weekly experiments	Cleaning done by	Range of total viable counts/swabbed area: Before cleaning 22° C.	Before cleaning 37° C.	After cleaning 22° C.	After cleaning 37° C.
			Object swabbed: top surface of blade			
1	8	Shop staff	105,000 to 10,500,000	53,750 to 7,875,000	5,125 to 275,000	3,500 to 146,250
1	4	R.J.G.	91,250 to 12,625,000	46,250 to 8,625,000	750 to 36,500	750 to 20,000
2–8	1	*	33,750 to 20,000,000	15,000 to 10,250,000	*	*
9, 10	1	R.J.G.	13,750 to 250,000	12,500 to 127,500	< 500 to 1,500	< 500 to 1,125
			Object swabbed: bottom surface of blade			
1	8	Shop staff	387,500 to 5,750,000	140,000 to 4,625,000	27,500 to 337,500	13,750 to 221,250
1	4	R.J.G.	103,750 to 5,000,000	90,000 to 2,250,000	< 500 to 13,500	< 500 to 7,625
2–8	1	*	15,000 to 9,125,000	13,750 to 3,000,000	*	*
9, 10	1	R.J.G.	70,000 to 400,000	55,000 to 225,000	500 to 2,125	750 to 1,250

* No results available.

Klebsiella sp. and *Proteus* sp. were also isolated. No attempt was made to isolate salmonellas as these organisms are not usually found in cooked or canned meats, but on five occasions coagulase-positive staphylococci were found. The viable counts were greatly reduced after the cleaning procedure. The results indicate also that the cleaning carried out by one of the authors (R.J.G.) was more efficient than that of the shop staff. There was still a wide range of bacteria found but coagulase-positive staphylococci, *Klebsiella* sp. and *Proteus* sp. were not isolated.

Table 5 shows the range of plate counts from swabbed areas of carving knives and can-openers in various premises after normal use and after the given cleaning procedure. All the carving knives examined had been used, before swabbing, for cutting and slicing various types of cooked and canned meats. All the can-openers examined had been used, before swabbing, for opening large containers (3–6 lb.) of canned meat. The results indicate that the cleaning procedure markedly reduced the total viable count and that cleaning by one of the authors (R.J.G.) was more efficient than that of the shop staff.

Table 5. *Range of bacterial plate counts at 22° and 37° C. from swabbed areas of carving knife blades and can-openers before and after cleaning*

Premises	No. of weekly experiments	Cleaning done by	Range of total viable counts/swabbed area: Before cleaning, 22° C.	Before cleaning, 37° C.	After cleaning, 22° C.	After cleaning, 37° C.
			Object swabbed: carving knife blades			
1	8	Shop staff	60,750 to 9,000,000	95,000 to 5,500,000	750 to 77,250	500 to 107,500
1	4	R.J.G.	62,500 to 10,000,000	55,000 to 8,750,000	< 500 to 1,250	< 500 to 2,000
2–7	1	*	17,750 to 3,000,000	10,250 to 2,150,000	*	*
9	1	R.J.G.	95,500	55,000	1,250	1,250
			Object swabbed: can-openers			
1	8	Shop staff	8,500 to 3,500,000	6,500 to 3,250,000	500 to 12,500	< 500 to 7,500
1	4	R.J.G.	42,500 to 350,000	15,000 to 200,000	< 500 to 750	< 500 to 1,750
3–4, 6–8	1	*	10,000 to 100,000	5,000 to 65,000	*	*
9, 10	1	R.J.G.	65,000 to 4,250,000	42,500 to 1,750,000	4,500 to 5,000	3,000 to 4,500

* No results available.

The concentration of the detergent/disinfectant used at premises 1, 9 and 10 was 0·75 %. The results of the minimum inhibitory concentration tests in Table 6 showed this to be satisfactory. The difference in temperature at the time of inoculation did not affect the results. The in-use test plates showed no growth at all.

Table 6. *Minimum inhibitory concentration tests with the commercial detergent/disinfectant used in premises* 1, 9 *and* 10

Organism	Laboratory reference no.*	Concentration of powder in cleaning solution† Inoculated at 22° C. 0·75 %	0·375 %	0·187 %	0·093 %	Inoculated at 45° C. 0·75 %	0·375 %	0·187 %	0·093 %
Coagulase-positive staphylococcus	CI/62/10312	−	−	−	+	−	−	−	+
	CI/62/10701	−	−	−	+	−	−	−	+
	CI/62/12146	−	−	−	+	−	−	−	+
	CI/62/12148	−	−	−	+	−	−	−	+
	FH/67/8006	−	−	−	+	−	−	−	+
Salmonella typhi									
NCTC 786	—	−	−	+	+	−	−	+	+
NCTC 3390	—	−	−	+	+	−	−	+	+
S. paratyphi B	FH/67/4244	−	+	+	+	−	+	+	+
S. typhimurium phage type 15a	FH/67/2723	−	−	+	+	−	−	+	+
S. typhimurium (untypable)	FH/67/8101	−	−	+	+	−	−	+	+
S. eimsbuettel	FH/67/8415	−	−	+	+	−	−	+	+
S. panama	FH/67/8422	−	−	+	+	−	−	+	+
S. worthington	FH/67/8910	−	−	+	+	−	−	+	+
Bacillus sp.	FH/68/164	−	−	−	+	−	−	−	+
Micrococcus sp.	FH/68/166	−	−	−	+	−	−	−	+

− = No growth; + = growth.
* CI, Cross Infection Reference Laboratory, Colindale. FH, Food Hygiene Laboratory, Colindale.
† Concentration of powder in cleaning solution used at premises 1, 9 and 10 was 0·75 %.

DISCUSSION

There is a risk of cross-contamination between meats sliced by a machine. As many different meats may be sliced during the day, a contaminated product can constantly recontaminate the machine, thereby increasing the spread of the organism to other meats. In this way the number of persons infected can ultimately exceed those who consumed the original contaminated meat. Such an accident was likely in the Aberdeen typhoid outbreak (Report, 1964). Three hundred and sixty-two persons who contracted typhoid fever had eaten or purchased food from the cold-meat counter in the supermarket involved. Of these only 131 were known to have eaten or purchased corned beef, 138 were known to have eaten or purchased unspecified cold meats, but 93 were known to have eaten or purchased cold meats excluding corned beef. Slicing of meats at the supermarket was done on one of the two slicing machines available for this purpose or by knife.

Cross-contamination may also occur when contaminated can-openers and carving knives are used. Couper, Newell & Payne (1956) reported that in the Pickering typhoid outbreak (1954–5) thirty-two persons contracted typhoid fever after eating ox tongue. One other person contracted this fever after eating ham known to have been cut by the same knife used for slicing the ox tongue.

Unopened cans of meat such as corned beef and ox tongue submitted to the Food Hygiene Laboratory for bacteriological examination are usually sterile or contain < 500 organisms/g. Cans of ham are an exception, however, as these products only receive a pasteurization treatment. It is interesting therefore to study the results of the bacteriological examination of various sliced samples of cooked and canned meats in this laboratory (Table 7). All of the 147 samples were obtained by Public Health Inspectors from shops, supermarkets, cafés, restaurants, public houses and hospitals in three London Boroughs. Some of the samples contained faecal coliforms or coagulase-positive staphylococci and 53 (36 %) of samples gave general colony counts at 35° C. of $> 10^6$ organisms/g. Such high counts can be attributed only to poor hygienic measures such as unrefrigerated storage conditions or contamination from personnel and machinery in the premises concerned. High counts from 50 (18 %) of 282 samples of various cooked and canned meats have been reported by Hughes (1960).

Table 7. *Results of bacteriological examination of cooked and canned meats submitted to the Food Hygiene Laboratory for examination* (*Jan.* 1967–*Feb.* 1968)

(Figures in parentheses indicate percentages.)

	Ham	Pork luncheon meat	Corned beef	Roast pork	Ox tongue	Beef (brisket and silver-side)	Spam
No. of samples examined	93	22	10	7	6	5	4
No. of samples known to have been cut by							
Slicing machine	46	12	7	3	3	1	3
Knife	4	0	1	1	0	1	0
No. of samples containing							
Non-faecal coliforms	39 (42)	3	4	4	2	3	1
Faecal coliforms (*E. coli*)	19 (20)	0	2	2	2	2	0
Coag.-pos. staphylococci	14 (15)	0	0	1	1	0	0
No. of samples with general colony count/g. at 35° C.							
10^5–10^6	30 (32)	9 (41)	2	1	0	2	0
$> 10^6$	37 (40)	4 (18)	0	6	5	1	0

The Food Hygiene Regulations (1960) require that equipment used in connexion with food should be kept clean. Our results clearly indicate that the bacterial counts from slicing machines, carving knives and can-openers are often very high. Similar results have been reported by C. A. Bailey (personal communication) from thirty-three swabs taken from slicing machines and twenty-eight swabs taken from knives. Ideally all equipment should be cleaned thoroughly each time it is used, but this is obviously not practicable in so far as it would delay the serving of customers. Cleaning twice daily should be possible in all food premises. Some slicing-machine manufacturers are now recommending the use of certain oils for regular and efficient cleaning of their machines. An assessment of these products is now being made and will be published separately, but the results to date are disappointing. The use of disposable paper and freshly laundered cloths instead of cloths disinfected overnight, as used in the present study, is also being investigated.

The results of the minimum inhibitory concentration tests showed that, under the conditions of the test, the concentration of detergent/disinfectant in the cleaning solution was adequate. However, during the cleaning operations the time of contact between the solution and the organisms present is so short that little reliance can be placed on the disinfectant action. It is the detergent action of the cleaning solution which is all-important in removing the organisms present by loosening the coating of fat which holds them on the surface of equipment. Methods for assessing the cleaning and bactericidal efficiencies of detergents for use in the catering industry have been given by Hobbs *et al.* (1960). The Food Hygiene Regulations (1960) are rather widely drawn and no specific direction on methods of cleaning or on suitable detergents and disinfectants are given. It is unfortunate that disinfectants used in the food industry are often described as 'sterilants' or 'sterilizing agents'. None of these agents can in fact produce sterility. We do not, however, condemn the use of combined detergent/disinfectants but do condemn complete reliance on any disinfectant action. No attempt was made to test the effect of using a disinfectant after cleaning with a detergent as this was not a common practice in the establishments visited, because the managers considered that this would increase the time of cleaning. Our results indicate that the cleaning procedure and detergent/disinfectant used in the present study was satisfactory but the counts shown are still not as low as would be desirable.

The absence of all growth on the in-use test plates does not indicate that the cleaning solution was sterile after use, but that it contained less than 100 viable organisms/ml. A test of this kind, carried out at regular intervals, can be useful for the maintenance of cleaning cloths in a hygienic condition.

Several methods for testing the efficiency of a cleaning procedure have been described. The agar-sausage technique (Ten Cate, 1963, 1965) has been recommended as a simple method which is useful for Public Health Inspectors (Greig, 1966) and for hygienic control in the factory (Thomas, 1967). The sticky-film method introduced by Thomas (1961) as a diagnostic aid in dermatology has been used successfully by Mossel, Kampelmacher & van Noorle Jansen (1966), who compared the method with the agar-sausage technique and the direct swabbing method with calcium alginate swabs to verify adequate cleaning of wooden surfaces. Recovery of organisms from surfaces was significantly greater by the direct swabbing method. However, for regular inspection purposes the agar-sausage method is convenient especially when laboratory facilities are limited or absent.

RECOMMENDATIONS

I. All food handling equipment should be cleaned at least twice daily. For slicing machines, carving knives and can-openers cleaning at midday and in the evening would not seem an unreasonable minimum aim. Where possible it is also desirable that a whole can of meat be sliced at one time and the equipment cleaned again before re-use.

II. Too much reliance on the attributes of commercial bactericidal detergents or detergent/disinfectants may lead to false security and staff must be reminded that effective cleaning procedures depend on their personal effort.

III. Slicing machines are potentially dangerous to the personnel using them and stricter safety measures are desirable.

IV. The design of commercial can-openers should be modified to make efficient cleaning possible.

V. Large canned hams, and all cooked meats and canned meats after slicing, should be stored at below 5° C.

VI. Although the present work provides no relevant evidence, it is recommended that cooked and canned meats be sliced on a separate machine from that used for cured products such as bacon, since cooked and canned meats are unlikely to receive further cooking.

VII. Food hygiene education should be extended so that all staff, including managers and directors, are fully aware of its importance and necessity.

SUMMARY

Experiments have been made in several supermarkets, shops and cafés to determine the bacterial counts on slicing machines, carving knives and can-openers after contact with various cooked and canned meats, and to find a simple, quick and effective cleaning method for such articles of equipment. The importance of personal effort in cleaning rather than a reliance on the known attributes of detergent/disinfectants is stressed. The methods available for testing the efficiency of cleaning procedures are outlined.

In vitro tests have shown that the detergent/disinfectant used in the present study at a concentration of 0·75% (w/v) was satisfactory. This concentration was the minimum inhibitory concentration for *Salmonella paratyphi B*, which was the most resistant of fifteen strains of bacteria studied.

The design of slicing machines and can-openers is discussed in relation to safety in use and ease of cleaning. Recommendations concerning the necessity of regular and effective cleaning of food-handling equipment and storage of cooked and canned meats before and after slicing are given.

We thank Dr Betty C. Hobbs and Dr J. C. Kelsey, the Directors of our laboratories, for their help and encouragement throughout the work and compilation of the paper. Such a paper would not have been possible without the full co-operation of the directors, management and staff of the premises visited and several Public Health Inspectors. We are grateful to Mr C. A. Bailey, Chief Public Health Inspector, Hatfield Rural District Council, for his help and permission to quote some of his unpublished work.

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J. Hyg., Camb. (1968), **66**, 451
With 1 *plate*
Printed in Great Britain

The immune response to viruses in calves

I. Response to Murray Valley encephalitis virus

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INTRODUCTION

Although the sequence of physically different antibodies in the immune response has attracted considerable attention, only relatively few animal-antigen systems have been subjected to detailed study. Uhr & Finkelstein (1967) have reviewed the work of Uhr and co-workers on the response to bacteriophage in guinea-pigs. Svehag & Mandel (1964*a*, *b*) characterized antibodies following inoculation of poliovirus in rabbits. Nossal, Austin & Ada (1965) analysed the response to *Salmonella* flagella antigen in rats. There has been very little significant work on the response in cattle, except for the study of the response to *Anaplasma marginale* by Murphy, Osebold & Aalund (1966). The differences between the responses in these systems indicates the importance of further detailed studies of a variety of animal-antigen systems.

The primary response to arboviruses in man shows the rapid development of neutralizing and haemagglutination-inhibiting (HI) antibodies; haemolytic complement fixing (HCFT) antibodies appear later and are usually relatively short lived (Southam & Green, 1958; Buescher *et al.* 1959; Scherer *et al.* 1959). In the secondary response there is a rapid increase of antibodies detected by all three serological tests and reinfection with a different but related arbovirus causes a broadly cross-reacting response in which antibodies are detected to other viruses which would not be detected in the primary response to either infecting virus (Theiler & Casals, 1958; Porterfield, 1962; Pond *et al.* 1967).

Bovine immunoglobulins have not been fully characterized although there is evidence for an immunoglobulin in addition to IgG and IgM which may be a bovine IgA (Murphy, Osebold & Aalund, 1965; Pierce & Feinstein, 1965; Winter, 1966). This paper describes the response in calves following experimental inoculation of Murray Valley encephalitis virus (MVE) using different serological tests and techniques of protein separation to study the part played by physically different immunoglobulins.

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MATERIALS AND METHODS

Serology

The HI test was carried out by the method of Clarke & Casals (1958), using kaolin adsorption to remove non-specific inhibitors (NSI). A modified direct HCFT as described by Boulanger & Bannister (1960) was used. This test will be described in more detail elsewhere (Sanderson, to be published). The plaque reduction (PR) test was carried out on the PS cell line (Westaway, 1966). This technique and some of the difficulties encountered with the assay of bovine antibody to MVE will be reported elsewhere (Sanderson, to be published).

Antibody separation

(i) Zone centrifugation was carried out by the method of Kunkel (1960). Sucrose gradients from 40 % to 10 % were produced in a gradient device (Britten & Roberts, 1960). This technique gave a complete and reproducible separation of IgM, IgG and NSI (Sanderson, 1968*b*). The amounts of IgG and IgM are expressed as the total HI units in the appropriate fractions when twenty fractions were collected.

(ii) Gel filtration in a $45 \times 3{\cdot}5$ cm. column of Sephadex G 200 (Pharmacia, Uppsala, Sweden) equilibrated in 1·0 M-NaCl in 0·02 M phosphate buffer, pH 8·0, was used to separate the proteins in 3 ml. of serum. The exclusion peak contained the IgM, the natural agglutinins and the NSI (Sanderson, 1968*b*).

(iii) Anion-exchange chromatography was carried out on DEAE Sephadex A 50 (Pharmacia). A 0·02 M pH 8·0 phosphate buffer was used to separate IgG into slow (IgG_S) and fast (IgG_F) fractions. Fractions containing IgG freed of IgM and NSI by gel filtration were dialysed against starting buffer and passed through a 10×2 cm. column of DEAE Sephadex. The fall through peak (IgG_S) was collected. The adsorbed protein containing IgG_F was eluted with 0·35 M phosphate buffer pH 8·0. These fractions were concentrated by dialysis against polyetheleneglycol (carbowax MW = 20,000) and then dialysed against phosphate buffered saline (PBS). They were then made up to the original serum volume (3 ml.). If the adsorbed proteins were eluted with a linear gradient from 0·02 M to 0·35 M phosphate buffer two peaks were resolved. Plate 1 *a* shows the analysis of these peaks by disk electrophoresis. Peaks 1 and 2 show the separation of IgG into slow and fast fractions. Immunoelectrophoresis also demonstrated this separation.

(iv) Disk electrophoresis was used analytically as in Plate 1 *a* or as a small-scale preparative electrophoretic technique to examine the properties of the antibody activity. This technique on acrylamide gel was essentially that of Davis (1964), with the following minor modifications. Gels were formed in glass tubes of 10 mm. internal diameter, 120 mm. in length. A 1 cm. spacer gel of 3·5 % acrylamide was formed over a 10 cm. separation gel of 7 % acrylamide. The tris-glycine pH 8·9 electrode buffer and tris-HCl gel buffer of Davis were used. A few drops of concentrated bromophenol blue were placed in the upper electrode chamber. The density of a 0·4 ml. sample was increased by adding 10 % sucrose and the sample layered on the gel under the electrode buffer. A current of 5 mA. per tube was applied until the sample had entered the spacer gel; the current was then increased to 10 mA. per tube until the bromophenol blue had reached the bottom of the tube gel. For antibody assay the gels were removed and sliced transversely (the spacer gel was not sliced) into disks weighing approximately 0·2 g. These were macerated with 0·4 ml. of PBS and allowed to stand overnight before titrating the supernatant for antibody activity. Alternatively gels were stained as described by Davis, although in this case the serum or serum fractions were diluted 1/20 before applying to the gel.

Experimental animals

Calves were purchased at local sales. They were of mixed dairy breeding and had been raised close to Brisbane, but no further details on their history were available. Their ages were estimated by physical appearance and so can only be regarded as approximate: calf 95, aged 5 months; 96, aged 5 months; 97, aged 3 months; 98, aged 3 months. The calves were maintained in insect-free housing.

Each calf was given a subcutaneous injection of 3 ml. of MVE infected unweaned mouse brain homogenate (containing approximately 10^8 LD50/0·1 ml. in unweaned mice). Calves 96 and 98 were given a second dose of the same material 34 days after the first to elicit a secondary response. Details of the virus strains have been given elsewhere (Sanderson, 1968*b*). Another group B arbovirus, strain MRM 3929 (R. L. Doherty, personal communication) was used in the fourth unweaned-mouse brain passage.

RESULTS

Calf 95

Antibody detected by the HI test approximately paralleled antibody detected by the PR test (Fig. 1). The HCFT antibody appeared only slightly later than HI antibody and reached maximum titre by the 8th day. Examination of the samples after zone centrifugation showed that up to the 10th day IgM was the major antibody present. After this it decreased and was not detectable by the 18th day. IgG was present, although in lesser amounts, as early as the 7th day and by the 15th day it was present in greater amounts. The HCFT antibody paralleled the IgG. Relatively little IgG_S was formed; it reached a maximum titre of 8, whereas the IgG_F reached a maximum titre of 160. The HCFT titre was less than 2 in the IgG_S fractions.

Calf 96

This calf (Fig. 2) showed a similar response to calf 95 as detected by the HI, HCFT, and PR tests. The second injection of MVE caused a drop in HI titre which reached its lowest level 2 days after the injection. This was followed by a classic secondary response with an increase in antibody detected by all three tests. Maximum HI and HCFT titre was reached by the 6th day but by the 10th day it was declining rapidly. After the 13th day samples were taken at 1 month intervals for 12 months. There was no significant fall in HCFT titre relative to HI titre; antibody detected by all tests fell to a very low level rapidly. Antibody was detectable by HI for only 8 months.

Zone centrifugal analysis showed that the first antibody detectable was IgG, and although a distinct IgM peak occurred, the IgG was at all times present at higher titre than IgM. Although it is difficult to compare the responses in the two calves because of the limited number of samples collected at this early stage of the response, it appeared that the IgG in calf 96 reached detectable levels earlier than

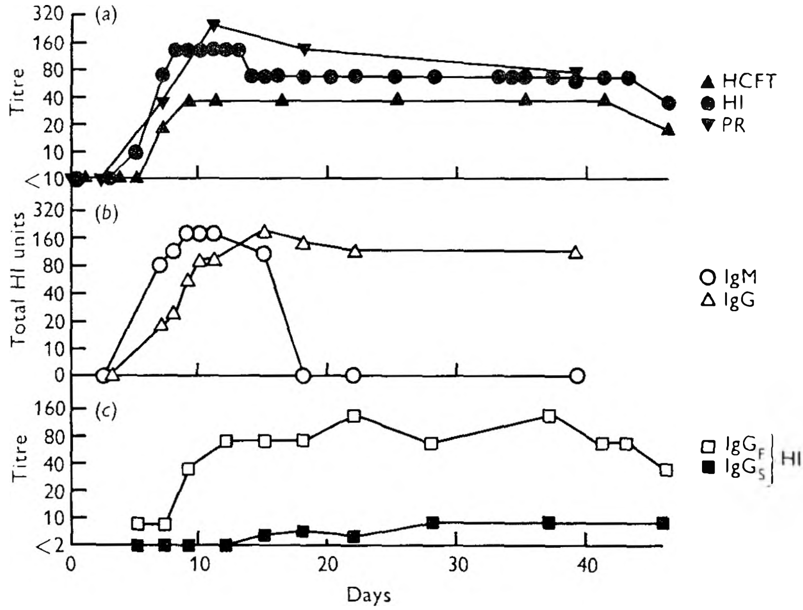

Fig. 1. Response in calf 95. (*a*) Total antibody response (whole serum). (*b*) Total HI antibody in IgM and IgG sucrose gradient fractions. (*c*) Antibody in IgG_F and IgG_S after separation on DEAE Sephadex.

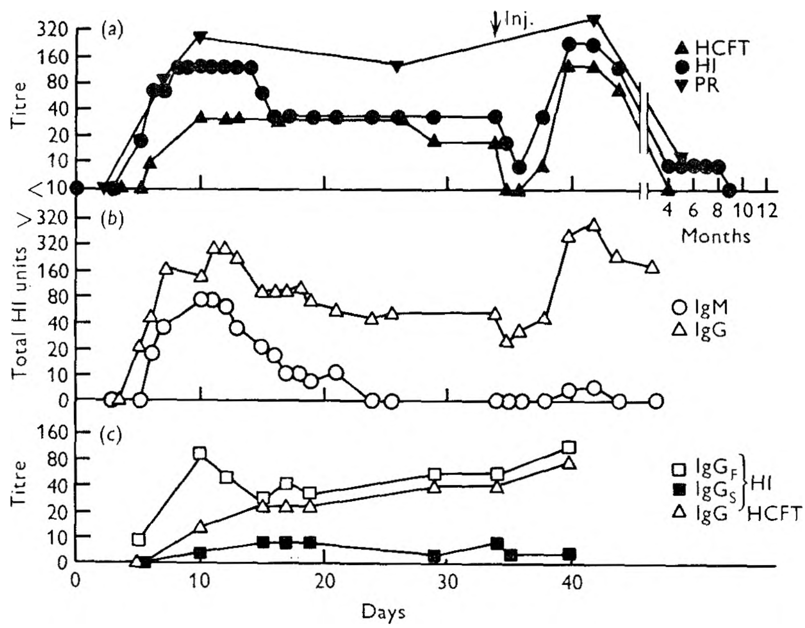

Fig. 2. Response in calf 96. (*a*), (*b*) and (*c*) as in Fig. 2.

in calf 95 and reached a higher titre. On the other hand, the IgM in calf 96 did not reach the same peak titre as in calf 95 although it was detectable for a longer period. In the secondary response calf 96 formed very little IgM; at its highest point only a total of six HI units were detected in the sucrose gradient.

Most of the HI and all of the HCFT antibody in both the primary and the secondary response was IgG_F. The IgG_S reached a maximum HI titre of 8, but the HCFT titre was at all times less than 2 (there was no anti-complementary activity in these fractions).

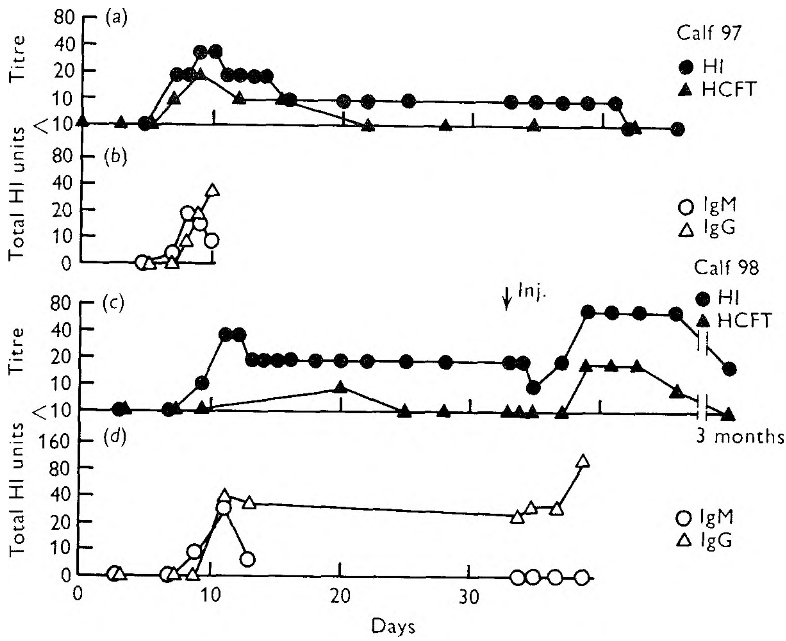

Fig. 3. (*a*) Total antibody response (whole serum) in calf 97. (*b*) IgM and IgG response in calf 97. (*c*) Total antibody response (whole serum) in calf 98. (*d*) IgM and IgG in calf 98.

Calf 97

The response in this animal (Fig. 3) was lower in titre than in calves 95 or 96. HCFT antibody was detected on the same day as the first HI antibody. A low titre of IgM was detected on the 7th day, and on the 8th day IgG had also appeared. Serum samples taken 12 and 28 days after inoculation contained only IgG_F antibody activity.

Calf 98

This animal (Fig. 3) showed a similar low-level response. An HI titre of 20 was maintained until the 34th day when a second injection was given. After this the HI titre fell slightly on the 2nd day, and then increased by the 4th day, reaching a maximum titre of 80 by the 6th day. Only a low level of HCFT antibody was detectable in the primary response; it appeared about 6 days after the second inoculation. IgM was first detected on the 9th day and reached a maximum about the 11th day and decreased rapidly. No IgG was detected on the 9th day but it had reached a maximum titre by the 11th day. No IgM was detected in the secondary response. Serum samples 12 and 28 days after the first inoculation contained only IgG_F.

IgM complement-fixing antibody

Sucrose gradient fractions from calves 95 and 96 containing relatively high levels of IgM antibody were dialysed against PBS, concentrated and tested by HI and HCFT. Several samples with HI titre from 32 to 128 were tested but none showed any complement fixation, although they had an anti-complementary titre of 4 to 16 which may have masked a low level of fixation. It was clear from these tests that the HCFT was at least 16-fold less sensitive than the HI test in detecting IgM.

Electrophoretic properties of IgG_F

To examine the electrophoretic nature of the material regarded as IgG_F, fractions were titrated after electrophoresis. All the serum samples separated by anion exchange chromatography from calves 95 and 96 and the samples at 12 and 28 days from calves 97 and 98 were examined, and within the limitations of the technique there was no indication of heterogeneity. The distribution of both HI and HCFT antibody was at all times similar to the distribution of HI antibody shown in Plate 1*b*.

Table 1. *Cross-reactions in MVE antiserum*

	Days after injection	HI titre* MRM 3929	Kunjin	MVE	Type of antibody
Calf 95	7	20	40	80	Whole serum (IgM)
	10, 15	40	20	160	Pooled sucrose gradient (IgG)
	42	40	20	160	Whole serum (IgG)
Calf 96	5	< 20	< 20	40	Whole serum (IgG)
	10	80	40	160	Whole serum (IgG and IgM)
	10	40	< 10	80	Heated whole serum (IgG)
	10, 11	< 20	40	80	Pooled sucrose gradient (IgM)
	42	320	80	1280	Whole serum (IgG)

* Samples early in the response were also tested for reactions to Kokobera, Stratford and Edge Hill. They were all less than 20, and are therefore not listed.

Cross-reactions

These are shown in Table 1. In calf 95 the first antibody formed was IgM when whole serum showed cross-reactions (HI) with Kunjin and MRM 3929 viruses, but not with Kokobera, Stratford or Edge Hill. Heating at 64° C. for 30 min. destroyed the HI activity, confirming that the reactions were due to IgM (Sanderson, 1968*b*). IgG antibody pooled from sucrose gradients of samples taken 10 and 15 days after inoculation also showed cross-reactions to the same viruses although to different titres, as did a whole serum 42 days after inoculation when IgG was the only antibody present.

In calf 96 the pre-inoculation sample contained no antibody to any of these six group B viruses. The first antibody detected was IgG and 5 days after inoculation this antibody reacted only with homologous virus antigen. At 10 days whole serum cross-reacted with both Kunjin and MRM 3929, but not with any of the other viruses. After heating this sample to inactivate IgM, the cross-reactions to Kunjin disappeared and thus the IgG reacted only with MVE and MRM 3929. Pooled IgM from sucrose gradients indicated that this immunoglobulin reacted more to Kunjin than to MRM 3929. At the peak of the secondary response when only IgG was present, whole serum cross-reacted with Kunjin but to higher titre to MRM 3929.

DISCUSSION

With the exception of calf 96 the first antibody to appear was IgM, which was rapidly followed by IgG, This explains why there was very little delay in the appearance of HCFT antibody. A clear biphasic response as has been reported in most other systems (Uhr, 1964; Svehag & Mandel, 1964*a*; Nossal *et al.* 1965) did not occur in the bovine response to MVE.

There was very little synthesis of IgM in the secondary response. The part played by IgM in the secondary response has been the subject of considerable discussion (Uhr & Finkelstein, 1967). Svehag & Mandel (1964*b*) found that the induction period, rate and duration of synthesis of IgM was similar in both the primary and the secondary response to poliovirus in the rabbit; there was no immunological memory to IgM nor inhibition of synthesis by pre-existing antibody. On the other hand, Nossal *et al.* (1965) were able to define conditions where the additional antibody in the secondary response to flagellin in rats was entirely IgM. Finkelstein & Uhr (1964) showed that passively administered antibody inhibited the synthesis of antibody, particularly IgM; this inhibition was more effective with secondary response IgG. While this appears a satisfactory explanation for the observed lack of IgM in the bovine secondary response, the secondary response in young calves described in the second paper in this series (Sanderson, 1968*a*) also showed a virtual lack of IgM, even without pre-existing primary response antibody.

The primary response in calf 96 was unusual in that IgG was detected before IgM. Such a possibility was suggested by Schoenberg *et al.* (1965) in contrast to the claim by Nossal *et al.* (1964) that cells first form IgM and then switch to IgG synthesis. However, the possibility must be considered that the response in calf 96 was not a true primary response but influenced by a previous natural arbovirus infection. It would seem unlikely that it represented a secondary response to homologous virus because there was no detectable antibody in the pre-inoculation sample, and the response did not resemble the experimental secondary responses, which showed a virtual absence of IgM and different kinetics of antibody formation. The response may be secondary, following natural infection by a different but related virus; thus the earliest IgG may represent a secondary response to shared specificity, and the IgM and some of the later IgG a primary response specific for MVE.

This type of reaction has been studied in relation to antigenically related proteins (Dixon & Maurer, 1955; Wiegle, 1961; Gilden & Tokuda, 1963). It is also a well-known phenomenon with influenza viruses where vaccination with a new strain of virus results in antibody directed mainly towards the first strain encountered (Davenport & Hennessy, 1956; Webster, 1966). After reinfection with a related group B arbovirus, antibodies are formed which cross-react broadly within the group (see Introduction). In calf 96 there was no antibody in the pre-inoculation sample to any of the six group B arboviruses tested. Cross-reactions occurred to MRM 3929 and Kunjin in the early IgG, but to no greater extent than occurred in calf 95 (Table 1). No cross-reactions were detected to Stratford, Edge Hill or Kokobera at any stage of the response to the first inoculation of MVE. This suggests that it was not influenced by previous arbovirus infection.

These cross-reactions are also interesting in that IgM and IgG showed different degrees of cross-reactions to other viruses. In both calves 95 and 96 the IgG reacted more to MRM 3929 than to Kunjin, and the relative titres remained similar throughout the primary and secondary response. On the other hand the IgM from both calves reacted to higher titre to Kunjin than to MRM 3929. This is further evidence that the change from IgM to IgG does not represent a total or a random switch over of a proportion of cells from IgM to IgG synthesis, as this should result in IgM and IgG of similar specificity. The fact that there is a difference in specificity emphasizes the importance of defining the immunoglobulin type in the classification of micro-organisms by their antigenic relationships.

Most of the IgG activity was due to the electrophoretically fast IgG_F; the IgG_S had only low titre HI activity and no HCFT activity. The HCFT was only about twofold less sensitive than the HI test in detecting IgG_F, whereas it was at least 16-fold less sensitive in detecting IgG_S. This suggests first that there is a difference in biological activity between the two immunoglobulins, and secondly that the antigens must been have strongly basic; that is, carry a net negative charge at physiological pH (Sela & Mozes, 1966). There is as yet very little information on the physical properties of arboviruses; however, they are eluted from DEAE cellulose only at high ionic strength at pH 8 (Nicoli, 1965), suggesting that they have low pI; that is, a net positive charge at this pH. Thus the net charge on the virus particle is not as important in determining the type of antibody formed as it is with the smaller molecules used by Sela & Mozes (1966). This could be interpreted as evidence that the virus particles are broken down, separating the positively charged part (lipid?) from the negatively charged antigen (protein?) before making contact with the factors (cells?) determining the type of immunoglobulin formed.

Following the second inoculation of MVE there was a decrease in circulating antibody titre due to the combination of virus with antibody. This did not reach its lowest level until 2 days after the inoculation. Had this been caused by the injected virus the decrease in titre should have occurred within hours, which suggests that some multiplication of virus took place in the presence of circulating antibody.

SUMMARY

Following inoculation of Murray Valley encephalitis virus into calves there was an early transient IgM response. The appearance of IgG was only slightly later than the first appearance of IgM in three calves, and in one calf IgG was detected

before IgM. The secondary response was characterized by the more rapid appearance of IgG and the virtual absence of IgM. The IgG was of the electrophoretically fast type; there was an almost insignificant amount of antibody activity in electrophoretically slow fractions. IgM and slow IgG had no complement-fixing activity. IgM and IgG showed different cross-reactions to other group B arboviruses. The different cross-reactions, and the appearance of IgG before IgM in one animal suggests that the switch-over from IgM to IgG synthesis in the response is not the result of either a total or a random proportion of cells producing IgM, changing to IgG production. The fact that the IgG was almost exclusively of fast electrophoretic mobility suggested that the virus antigenic components were strongly basic, and as this is contrary to the chromatographic properties of arboviruses, it is suggested that the virus particles must be broken down before making contact with the factors determining the type of immunoglobulin formed.

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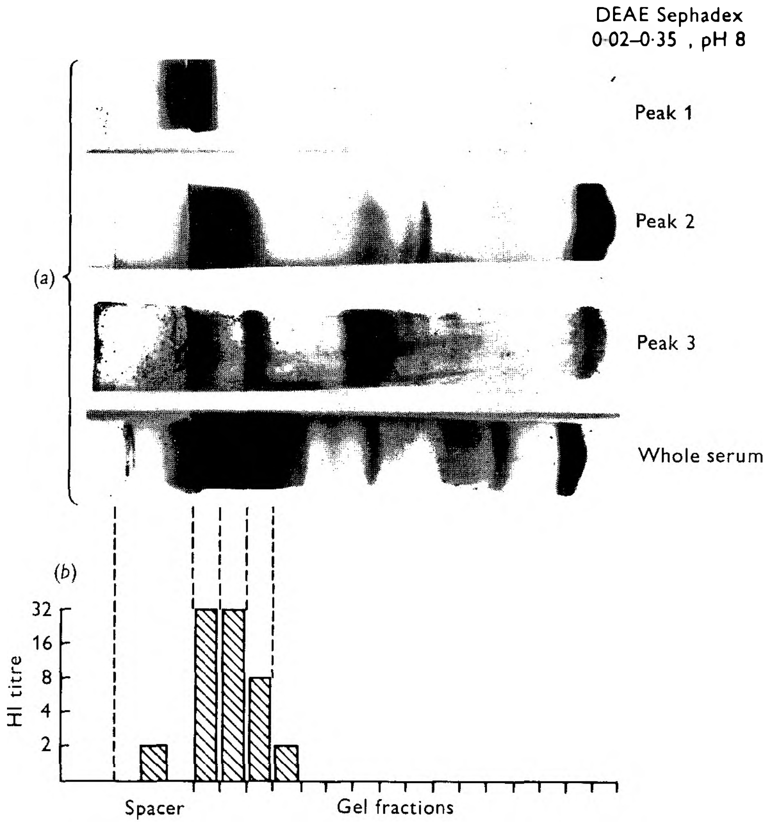
EXPLANATION OF PLATE

(*a*) Disk electrophoretic analysis of peaks 1, 2 and 3 from DEAE Sephadex chromatography, and whole bovine serum. (*b*) Titration of antibody eluted from gel segments corresponding to the stained gels of (*a*).


DEAE Sephadex
0·02–0·35 , pH 8
Peak 1
Peak 2
Peak 3
Whole serum
(a)
(b)
32
16
8
4
2
HI titre
Spacer
Gel fractions

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The immune response to viruses in calves

II. The response in young calves

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INTRODUCTION

There have been a number of studies carried out to determine the immune response in young animals: for example, using bacteriophage as antigen (Uhr *et al.* 1962; Silverstein *et al.* 1963; Kim, Bradley & Watson, 1966), bacterial antigens (Sterzl, *et al.*, 1964; Bellanti *et al.* 1963) and poliovirus as antigen (Svehag & Mandel, 1964*b*). In general, it has been found that the young animal produces an early IgM response with a relatively delayed IgG, and the responses are of lower magnitude than those of mature animals. Probably the one clear feature to emerge from this work is the variation between different species, and also between different antigens in any one species. Studies on young calves have been restricted to either field trials of vaccines (Henning, 1953) or experiments involving serological tests without antibody characterization (Lambert *et al.* 1961; Smith & Ingram, 1965; Dawson, Darbyshire & Lamont, 1965).

This paper describes experiments on the response to viruses in young calves using techniques of protein separation to study the part played by physically different immunoglobulins.

MATERIALS AND METHODS

The serological tests and techniques of protein separation have been described in the previous paper (Sanderson, 1968*a*).

Viruses

The group B arbovirus Murray Valley encephalitis (MVE) was used as infected unweaned mouse brain homogenate of 10^8 LD 50/0·1 ml. in unweaned mice; the dose was 2·5 ml.

The group A arbovirus Getah (strain N 544) was used as infected unweaned mouse brain homogenate of 10^5 LD 50/0·1 ml. in unweaned mice; the dose was 2·5 ml. Full details of these viruses are given elsewhere (Sanderson, 1968*b*).

Parainfluenza 3 virus (PI 3)—a strain from man (obtained from Commonwealth Serum Laboratories, Parkeville, Victoria)—was propagated in primary bovine kidney cells and was 10^6 TCID 50/0·1 ml.; the dose was 4 ml.

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Reovirus 1 (Reo)—Lang strain (Stanley, 1961)—was propagated in primary bovine kidney cells and was $10^{6\cdot5}$ TCID 50/0·1 ml.; the dose was 4 ml.

Experimental design

The principal of the experiment was to inject virus preparations at different ages over the first weeks of life, to collect bi-weekly serum samples over a period of 4 weeks for antibody assay, and then to give a second injection to test for a secondary response. To increase the amount of data obtained from a small number of animals four viruses were used at different times in each calf. The experiment is set out in Table 1. Eight calves were obtained from local sales. In most cases the ages were obtained from the owner, while the age of some was estimated from the condition of the umbilical cord and the general appearance. Neither of these could be considered completely reliable. A blood sample was taken and the calves given the first injection on the day after arrival. The eight calves were treated as pairs, and each pair was injected subcutaneously with a different virus at intervals of 7 days until after 4 weeks each animal had received an injection of the four viruses. Over the next 4 weeks the same sequence was repeated, so that each animal received a second injection of the same virus 4 weeks after the first. The calves were kept in insect-free housing.

Table 1. *Experimental design; calf identification number and age at each injection*

(Age in days shown in parentheses.)

Calf no.							
99	100	101	102	103	104	105	106
			First injection				
MVE	MVE	Reo	Reo	Get	Get	PI3	PI3
(12)	(4)	(8)	(8)	(6)	(11)	(11)	(11)
Reo	Reo	Get	Get	PI3	PI3	MVE	MVE
(19)	(11)	(15)	(15)	(13)	(18)	(18)	(18)
Get	Get	PI3	PI3	MVE	MVE	Reo	Reo
(26)	(18)	(22)	(22)	(20)	(25)	(25)	(25)
PI3	PI3	MVE	MVE	Reo	Reo	Get	Get
(33)	(25)	(29)	(29)	(27)	(32)	(32)	(32)
			Second injection				
MVE	MVE	Reo	Reo	Died	Get	PI3	PI3
(40)	(32)	(36)	(36)		(39)	(39)	(39)
Reo	Reo	Get	Get	—	PI3	MVE	MVE
(47)	(39)	(43)	(43)		(46)	(46)	(46)
Get	Get	PI3	PI3	—	MVE	Reo	Reo
(54)	(46)	(40)	(50)		(53)	(53)	(53)
PI3	PI3	MVE	MVE	—	Reo	Get	Get
(61)	(53)	(57)	(57)		(60)	(60)	(60)

This experimental design allowed a greater number of responses to be studied in a small number of calves, but also provided a considerable economy in the separation techniques, as each fraction could be tested with each of the viral antigens.

RESULTS

The expected total number of responses was not realized because all eight calves possessed maternally derived antibody to PI 3. Two calves (99 and 101) possessed maternal antibody to Reo. One calf (103) died without obvious clinical symptoms or gross pathological lesions. This occurred 5 days after an injection of Reo although there was no reason to believe that the two events were related. For these reasons only twenty-one responses were studied, consisting of eight to MVE, eight to Getah and five to Reo. In addition, the levels of maternal antibody to PI 3 and Reo were sufficiently low in one calf to allow a detectable response to the second injection. The presence of maternal antibody allowed a comparison of this immunoglobulin with autogenous antibody by the same techniques of physical separation.

Each serum sample was tested by the haemagglutination-inhibition (HI) test and the haemolytic complement fixation test (HCFT). Selected samples were then tested by HI after zone centrifugation; this technique allowed the detection of lower levels of antibody than the test on whole serum and so the interpretation of the experiment is based on these results. For this reason the results of the HI and HCFT on whole serum are not tabulated. The results are shown in Figs. 1–3.

Table 2. *Summary of the type of response after the first and second injection of virus, showing the types of immunoglobulin synthesized*

(Calf number with age at injection in days in parenthesis.)

	Response to first injection			Response to second injection		
Virus	IgM + IgG	Only IgG	No response	IgM + IgG	IgG	Primary type
MVE	99 (12)	—	—	—	99	—
	—	100 (4)	—	—	100	—
	—	—	101 (29)	—	101	—
	102 (29)	—	—	—	102	—
	103 (20)	—	—	—	Died	—
	—	—	104 (25)	104	—	—
	—	—	105 (18)	105	—	—
	—	106 (18)	—	—	106	—
Getah	—	99 (26)	—	—	99	—
	100 (18)	—	—	—	100	—
	101 (15)	—	—	—	101	—
	102 (15)	—	—	—	102	—
	—	—	103 (6)	—	Died	—
	104 (11)	—	—	—	104	—
	—	105 (32)	—	—	105	—
	—	—	106 (32)	—	—	106
Reo	100 (11)	—	—	—	100	—
	102 (8)	—	—	—	102	—
	104 (32)	—	—	—	104	—
	105 (25)	—	—	105	—	—
	—	—	106 (32)	—	106	—

Response to the first injection

After the first injection of each virus the calves reacted either by producing IgM followed by IgG, or by producing IgG without detectable IgM. In some no antibody was detected in the 28 days before the second injection (Table 2).

(i) In response to the first injection of MVE (Fig. 1), five of the eight calves formed detectable antibody but only three of these formed detectable IgM

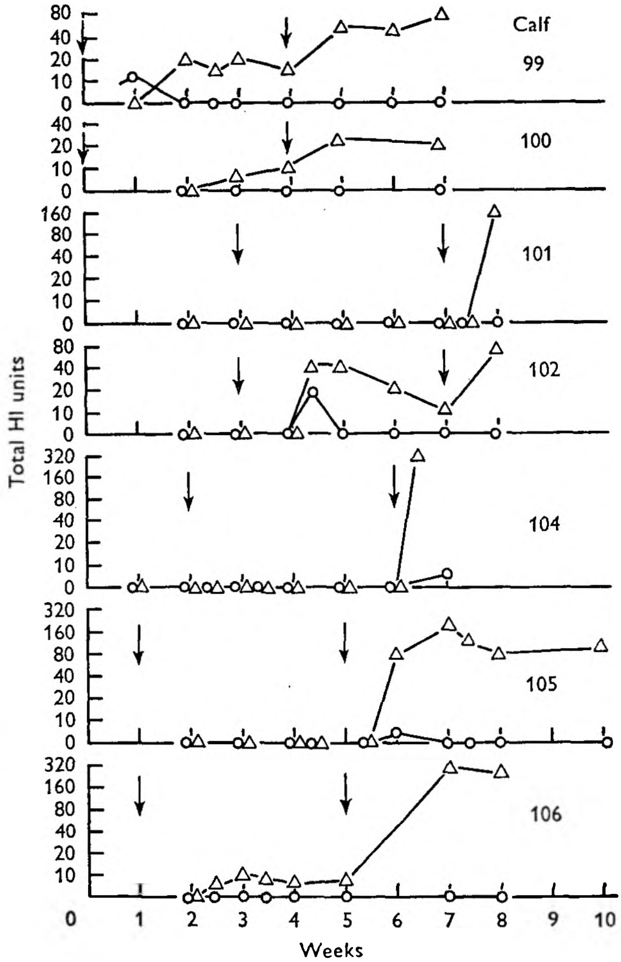

Fig. 1. Response to MVE shown as the total HI units in IgM and IgG fractions of sucrose gradients. Calf 103 is excluded. The arrows indicate the days on which virus was injected. All samples (bi-weekly) were tested for antibody in whole serum, in each case pre-inoculation samples did not contain antibody. △, IgG; O, IgM.

(Table 2); in two of these the IgM was low in titre and only persisted for a short time. The other animal (103) died before the completion of the experiment and so is not included in Fig. 1. The IgM was first detected by the 7th or the 10th day and IgG appeared by the 10th or 14th day (Table 3).

(ii) In response to Getah (Fig. 2) all but two calves formed detectable antibody and four of the six formed IgM (Table 2) which appeared by the 7th day in three and by the 17th in one calf (Table 3). In calf 100 IgM persisted for at least 3 weeks although in the others it was of short duration. The IgG was detected much later than had been the case with MVE, varying from 17 to 28 days after injection. The two calves not responding (103 and 106) are omitted from Fig. 2.

Table 3. *The number of days after each injection before HI antibody was detected, and the duration of IgM*

(Calf number with age at injection in parenthesis. —None detected.)

Virus	Calf no.	Injection no.	Primary response IgM (duration)	Primary response IgG	Secondary response IgM	Secondary response IgG
MVE	99 (12)	1st	7 (7)	14	—	7
	100 (4)	1st	—	14	—	7
	101 (29)	4th	—	—	—	7
	102 (29)	4th	10 (7)	10	—	7
	103 (20)	3rd	10	10	—	Died
	104 (25)	3rd	—	—	7	7
	105 (18)	2nd	—	—	4	7
	106 (18)	2nd	—	10	—	7
Getah	99 (26)	3rd	—	28	—	7
	100 (18)	3rd	7 (21)	21	—	3
	101 (15)	2nd	7 (7)	17	—	7
	102 (15)	2nd	7 (7)	21	—	7
	103 (6)	1st	—	—	—	Died
	104 (11)	1st	17 (7)	17	—	7
	105 (32)	4th	—	28	—	3
	106 (32)	4th	—	—	—	—
Reo	100 (11)	2nd	7 (21)	14	—	7
	101 (8)	1st	—	—	—	7
	102 (8)	1st	3 (18)	3	—	7
	104 (32)	4th	7 (7)	14	—	7
	105 (25)	3rd	7 (21)	7	7	7
	106 (25)	3rd	—	—	—	7

(iii) In response to Reo (Fig. 3) four of five calves formed IgM and IgG (Table 2). The response in 99 and 101 was complicated by maternal antibody and is not included in this table. The IgM was in general of higher titre than the IgM to the other viruses and appeared by the 7th day in three calves and by the 3rd day in one calf (Table 3). IgG was detected from 3 to 14 days after injection. As the IgG was usually detected well before the end of the 28-day period with all three viruses, it is unlikely that the lack of detectable IgG in the other calves was due to a response delayed beyond the 28 days of the experiment.

All except calf 106 produced IgM in response to at least one virus. This indicated that the ability to respond with IgM was diminished rather than deficient in individual calves. There was no observable relationship between the type of response and the age at injection or the injection sequence. None of the calves with maternally derived antibody formed IgM and there was no increase in IgG levels.

Response to the second injection

After the second injection a secondary response occurred in all but one case (Table 2). This was calf 106, which had not responded to the first injection of Getah and a low titre response of the primary type followed the second injection. The type of immunoglobulin involved in this response was not determined and so it is omitted from Fig. 2. Only three of the secondary responses contained IgM and even in these it represented a very small proportion of the total antibody.

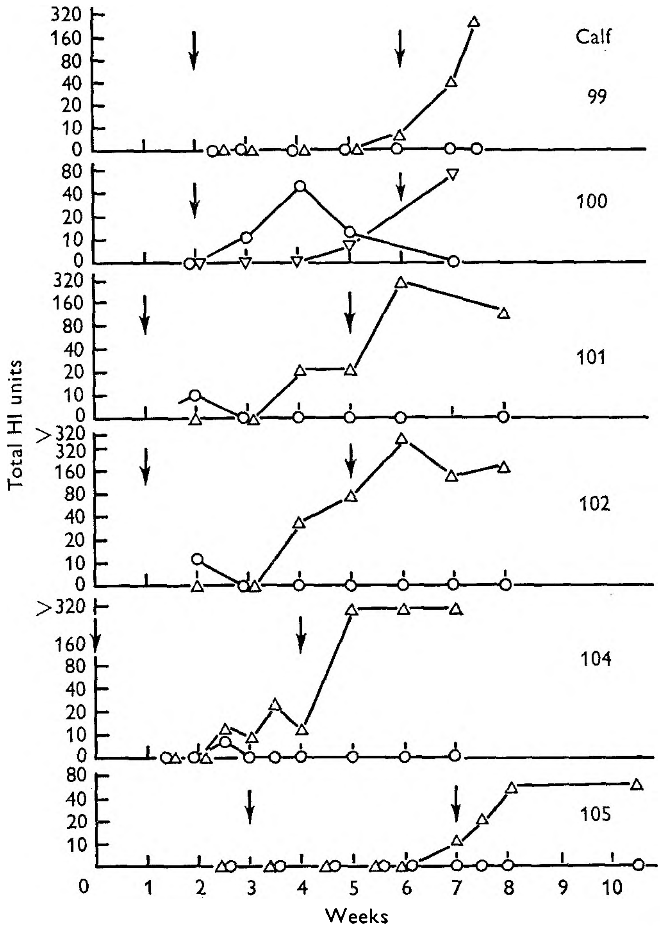

Fig. 2. Response to Getah (as in Fig. 1). Calves 103 and 106 excluded. △, IgG; ○, IgM.

In calf 101 the levels of maternally derived antibody to PI3 and Reo were low. The second injection of Reo gave a marked secondary response (Fig. 3), indicating that the passive antibody was of sufficiently low titre to allow priming for a secondary response. The second injection of PI3 caused a response which seemed most likely to be a primary response in that the antibody titre was of a low level on the 7th day (Fig. 3). There was no IgM detected in this response. In none of the other calves with passive antibody did the second injection result in a detectable increase in antibody.

To further examine the properties of the IgG at different phases of the response, various samples were examined by anion exchange chromatography and by electrophoresis on acrylamide gel. The serum samples tested were those taken at the following weeks after the beginning of the experiment: calf 99, 4 and 6; calf 100, 3 and 4; calf 101, 1, 4 and 6; calf 102, 1, 2, 5 and 6; calf 104, 5 and 6; calf 105, 3 and 6; calf 106, 3, 5 and 6. These times correspond to the weeks in Figs. 1–3.

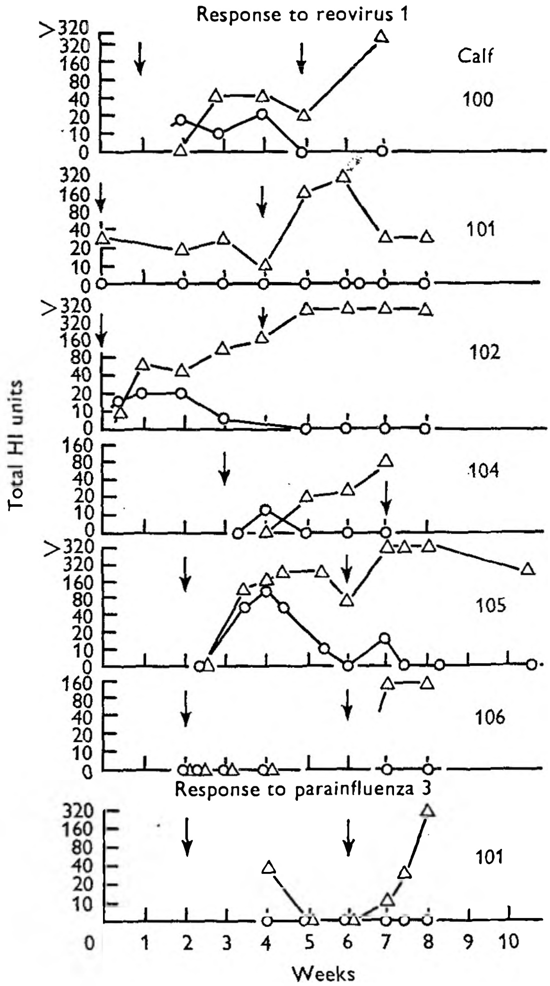

Fig. 3. Response to Reo (as in Fig. 1). Calf 101 was the only animal with pre-inoculation (maternal) antibody. Response to PI3 in calf 101; maternal antibody had disappeared by the fifth week and the animal responded to the second injection of virus. △, IgG; ○, IgM.

In only one sample was antibody detected in the IgG_S fractions (1/2 was the lowest dilution tested). This was in calf 102 during the secondary response to Reo (6th week) when the HI titre was 2560, and the titre in the IgG_S was 32. After separating by electrophoresis, the antibody of both maternal and autogenous origin in all samples was restricted to the IgG_F zone. That maternal antibody is IgG_F has been shown by Murphy *et al.* (1964) and Pierce & Feinstein (1965).

The IgG_S from calf 102 with an HI titre of 32 had no complement-fixing activity at a dilution of 1/2, suggesting that the differences in chromatographic properties reflected different biological properties.

After the secondary response most of the calves showed a marked drop in antibody titre, which in some cases fell to a very low level within a few months (Table 4).

The properties of antibodies to MVE were studied by the plaque reduction test, which indicated both neutralizing and plaque enhancing activity. These results are reported in detail elsewhere (Sanderson, to be published.)

Table 4. *Duration of antibody after second injection*

			Months after second injection								
Virus	Calf	Test	0	1	2	3	4	5	6	7	8
MVE	101	HI	160	160	40	40	20	—	10	—	—
		HCFT	40	40	10	10	< 10	—	< 10	—	—
		NT	6	—	—	—	0	0	0	—	—
	106	HI	160	80	80	40	10	—	—	—	10
		HCFT	40	40	40	—	< 10	—	—	—	—
		NT	6	6	—	4	3	—	—	—	1
Getah	101	HI	80	—	—	—	40	—	—	—	—
		HCFT	160	—	—	—	20	—	20	—	—
	106	HI	10	10	< 10	—	—	—	—	—	—
		NT	3	4	2	—	0	—	—	—	—
Reo	101	HI	160	10	< 10	—	—	—	—	—	—
		HCFT	40	< 10	—	—	—	—	—	—	—
	106	HI	160	10	< 10	—	—	—	—	—	—
		HCFT	160	—	—	—	—	< 10	—	—	—
PI3	101	HI	40	80	80	20	40	—	10	—	—
		HCFT	20	20	—	10	—	—	10	—	—

DISCUSSION

Thirty-two possible responses were given by the use of four different viruses in eight calves. This number was reduced to twenty-one by the death of one calf and the presence of maternal antibody to PI3 in all eight calves and to Reo in two calves.

Three types of response occurred after the first injection of virus. These consisted of either sequential synthesis of IgM and IgG, IgG with no detectable IgM or no response at all (Table 2). There was no IgM and no increase in IgG titre in the presence of maternal antibody. In one calf there was priming for a secondary response in the presence of low levels of maternal antibody.

The most surprising finding in this experiment was the virtual absence of IgM in the primary response to MVE and Getah in the young calves. Only one calf formed a significant amount of IgM in response to either of these viruses, this was calf 100 (Fig. 2) in response to Getah. In those cases in which IgM was produced it was usually detected by the 7th or 10th day, which was not greatly different from the response in the older calves of the previous paper. The time before IgG was first detected was in general much longer and more variable than the IgG response in older calves (Table 3). A relatively low level of IgM which was rapidly replaced by IgG at much higher titre was reported in the young rat (Williams, 1966), but in general the young animal is regarded as producing relatively more IgM (Uhr *et al.* 1962; Bellanti *et al.* 1963; Silverstein & Kraner, 1964; Sterzl *et al.* 1964; Buffe & Burtin, 1967).

In all the calf responses where IgM was detected it was followed by IgG, but in four cases IgG was detected without IgM and in most of the other responses the IgM was of a remarkably transient nature. This suggested that in calves at this age the induction requirements for IgG were lower than the requirements for the formation of IgM. The reverse situation seems to be the more general rule in the adult animal (Uhr & Finkelstein, 1963; Svehag & Mandel, 1964*a*, *b*), although Williams (1966) found that rats injected on the day of birth gave a secondary response to a later injection in the absence of a primary response.

In the secondary responses IgM was either not detected or occurred at very low levels, and this was the case in six secondary responses in which there was no detectable antibody in the primary response. This is different from the situation in rabbits, where similar amounts of IgM appear in both the primary and the secondary response (Svehag & Mandel, 1964*b*), and in rats where an increased amount of IgM can be demonstrated in the secondary response (Nossal, Austin & Ada, 1965).

The fact that calves can be immunologically primed without giving a serological response indicates that vaccine trials should be based on the ability to stimulate immunological memory. This state is tested where vaccination is measured by resistance to challenge. These results provide an explanation for the observation by Henning (1953) and Lambert *et al.* (1961) that vaccination of calves produced good field immunity but the serological response was of poor grade.

SUMMARY

Injections of virus in calves up to about 5 weeks of age caused either the production of both IgM and IgG, or the production of only IgG, or no detectable antibody. In all but one case priming for a secondary response occurred even in the absence of a detectable primary response. These results suggest that in the young calf immunological memory is more readily induced than IgG synthesis. IgM appeared by the 7th or 10th day, which was similar to the response in older calves. IgG was more variable in its appearances but was usually considerably delayed relative to the response in older calves. In general only low levels of IgM were formed in the primary response and it was virtually absent from the secondary responses. Higher levels of IgM were formed after reovirus inoculation than after Murray Valley encephalitis virus or Getah viruses. The delay in appearance of IgG was more pronounced in response to Getah than to reovirus or Murray Valley encephalitis virus. High levels of maternally derived passive antibody inhibited the development of an active response, although in one animal a response occurred in the presence of low levels of passive antibody.

I wish to thank Professor J. Francis and Dr P. B. Spradbrow for their interest in this work, and Mr I. Grindrod and Miss A. Muller for technical assistance.

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Further studies on the antibiotic resistance of *Shigella sonnei*

I. Transferable antibiotic resistance

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INTRODUCTION

Previous studies on the epidemiology of Sonne dysentery in the London area (Davies, 1954; Farrant & Tomlinson, 1966) described the trend towards an increased frequency of strains of *Shigella sonnei* resistant to sulphonamides and later to streptomycin and tetracycline. It seemed relevant to continue and extend these observations. Recent work on the transfer of antibiotic resistance between organisms of many genera within the family Enterobacteriaceae (Datta, 1962; Watanabe, 1963; Anderson & Lewis, 1965) prompted an attempt to determine how much of the antibiotic resistance of *Sh. sonnei* could be transferred to a suitable recipient strain of *Escherichia coli*.

MATERIALS AND METHODS

Source of cultures

The strains of *Sh. sonnei* examined were isolated in this laboratory from patients in the London area.

We have continued our policy of examining one strain from each incident—an incident being defined as a number of infections that might be expected to have a single source. In practice this has meant that one strain has been examined from each household or family. Day nursery outbreaks and cases in large residential institutions have been excluded from the analysis.

The methods used for the isolation and identification of *Sh. sonnei* were those described previously (Farrant & Tomlinson, 1966).

Antibiotic sensitivity testing

Antibiotic sensitivity was determined by the disk technique using Oxoid disks (sulphatriad 300 μg., streptomycin 25 μg., tetracycline 50 μg., ampicillin 25 μg., neomycin 30 μg., colistin 200 μg.) or disks prepared in the laboratory (chloramphenicol 30 μg.). Tests for sulphonamide sensitivity were carried out on lysed blood agar plates; antibiotic sensitivities were determined on nutrient agar plates. The inoculum for both was a loopful of a 4 hr. peptone water culture.

Minimal inhibitory concentrations (MIC) were determined by incorporating serial dilutions of the antibiotic in nutrient agar over the range 2–2000 μg./ml. and inoculating segments of a plate with a loopful of an overnight broth culture.

Abbreviations

Su = sulphonamide, St = streptomycin, Te = tetracycline, Amp = ampicillin.

Transfer of antibiotic resistance

Transfer experiments were carried out by growing the strain of *Sh. sonnei* in broth with *E. coli* K12 met$^-$ F$^-$ (K12). This mixture was usually incubated overnight but sometimes, to separate progeny of different resistance patterns, shorter incubation periods were used. The *E. coli* recipient was separated from the *Sh. sonnei* donor by plating the mixed culture on medium (minimal agar) which, while supporting the growth of K12, was nutritionally deficient for *Sh. sonnei*. Quantitative estimations of resistance transfer were performed by making serial dilutions of an overnight mixed culture. A loopful of each dilution was spread over an area of a plate of minimal agar with or without appropriate antibiotics (Anderson & Lewis, 1965). Individual colonies were picked from the minimal agar, purified and tested for antibiotic resistance. When the counts on plates containing two different antibiotics were similar and all the progeny picked from each medium were resistant to both antibiotics it was assumed that the resistance determinants were linked. If the counts on the two media were different and progeny resistant only to one antibiotic were isolated from the appropriate medium it was assumed that the resistances travelled independently. For example:

Sh. sonnei resistant to sulphonamide, streptomycin and tetracycline mated with K12 and plated on minimal agar:

Count on minimal agar,	10×10^6 cols./loop.
Count on minimal agar + Su,	5×10^4 cols./loop.
Count on minimal agar + St,	10×10^5 cols./loop.
Count on minimal agar + Te,	10×10^5 cols./loop.
Ten colonies picked from each medium:	
From minimal agar,	1 resistant to St and Te, 9 sensitive,
From minimal agar + Su	2 resistant to Su, St and Te, 8 resistant to Su,
From minimal agar + St,	10 resistant to St and Te,
From minimal agar + Te,	10 resistant to St and Te.

Interpretation: Su resistance transferred to about 0·5 % of recipients and travelling separately from a linked (StTe) resistance (approx. 10 % of progeny resistant).

Resistance determinants transferred as a single unit are indicated by the use of bracket, e.g. (StTe). Resistance determinants transferred independently are separated by commas, e.g. Su,Te.

Minimal agar was prepared from a double strength solution which could be stored; for use a volume of warmed solution was added to an equal volume of melted double strength agar (24 g. Davis agar/l.). Melting the complete medium proved deleterious. The double strength solution contained NaCl, 10·0 g.; $MgSO_4 7H_2O$, 0·4 g.; $NH_4H_2PO_4$, 2·0 g.; K_2HPO_4, 2·0 g.; lactose, 4·0 g.; dL methionine, 0·01 g.; nutrient broth, 0·4 ml. in a litre of distilled water; this solution was distributed in 100 ml. volumes and sterilized by steaming for 30 min.

Antibiotics, when required, were added to the complete medium just before it was poured into plates to give the following final concentrations; sulphathiazole 5 μg./ml., streptomycin sulphate 10 μg./ml., terramycin hydrochloride 10 μg./ml. and ampicillin 25 μg./ml.

RESULTS

Antibiotic resistance

The fluctuations in the antibiotic resistance of *Sh. sonnei* since this study was started in October 1956 are shown in Table 1. The time interval chosen, including the last quarter of one year with the first three-quarters of next, is appropriate to a disease such as Sonne dysentery in which the incidence gradually increases throughout the winter reaching a maximum in the spring and declining in the summer (Bradley, Richmond, Shaw & Taylor, 1958). The proportion of strains resistant to any particular antibiotic at any one time would be influenced by a large local outbreak producing a large number of strains of a particular pattern. This may account for some minor fluctuations but the strains examined in any season were sufficiently widely distributed in time and space for this not to be a serious source of error.

Table 1. *Antibiotic resistance of* Shigella sonnei 1957–67

Period (quarters)	No. of strains examined	% resistant to		
		Su	St	Te
1956/4–1957/3	556	31	0	1
1957/4–1958/3	1174	63	0	4
1958/4–1959/3	1412	81	0·5	12
1959/4–1960/3	1968	54	6	5·5
1960/4–1961/3	467	58	4	5
1961/4–1962/3	765	80	7·5	14
1962/4–1963/3	2052	90	10	13
1963/4–1964/3	618	92	31	40
1964/4–1965/3	608	95	20	31
1965/4–1966/3	759	93	45	24
1966/4–1967/3	533	82	60	12

It will be seen that most strains examined since the 1961 season have been resistant to sulphonamides. The proportion resistant to streptomycin has been gradually increasing; the proportion resistant to tetracycline has varied from season to season although we have not in recent years observed the low incidence of resistance which existed at the start of our study in 1957.

The sensitivity of the strains to other antibiotics has been tested over various periods.

Ampicillin. Since March 1966 all strains have been examined; some results were reported by Scrimgeour (1966). The proportion of resistant strains has varied from 70 % in the third quarter of 1966 to 95 % in the third quarter of 1967 (see Table 3).

Chloramphenicol. Strains were examined regularly from October 1962 to November 1963 (over 2000 strains). One strain was found to be resistant. No further testing of resistance to this antibiotic has been carried out.

Neomycin. The sensitivity of all strains has been determined since October 1962. Out of a total of more than 4500 strains only three resistant strains have been found.

Colistin. Since November 1964 all strains have been examined. No resistant strains have been found.

Transferable antibiotic resistance

During the first part of 1966 some preliminary work was carried out on the transferable antibiotic resistance of selected strains, usually those resistant to more than one antibiotic. The results are summarised in Table 2.

Table 2. *The transferable antibiotic resistance of selected strains of* Shigella sonnei

Resistance	No. of strains tested	No. of strains transferring resistance	% of progeny resistant
Su	261	256	0·1–50
St (MIC 800 μg./ml.)	118	0	—
St (MIC 16–256 μg./ml.)	87	81	10–60
Te	100	100	10–60
Amp	66	1	10

Sulphonamide. Most strains tested transferred this resistance to K12. Su resistance was usually transferred independently of other resistances and at a relatively low rate (approx. 0·1–1 % of progeny resistant). A few strains were encountered in which the Su resistance was transferred linked to St resistance (SuSt) or to St and Te resistance (SuStTe). In these strains the transfer of the combined resistance was more efficient, 10–50 % of the recipients receiving the resistance.

Streptomycin. St resistant strains of *Sh. sonnei* could be divided into those with an MIC of 800 μg./ml. or more and those with an MIC in the range 16–256 μg./ml. Those strains with a high level of resistance to St did not transfer this resistance to K12 although many of them had a transferable Su resistance and some a transferable Te resistance. It has been suggested by Watanabe (1966) that this high level resistance is chromosomal. The lower level St resistance was usually transferable and was frequently linked to other transferable resistances, (SuSt), (StTe) or (SuStTe).

Tetracycline. All strains which we examined transferred their tetracycline resistance either separately or linked to St or Su and St. No strain transferred a linked (SuTe) resistance.

Ampicillin. The only strain transferring ampicillin resistance had an MIC of more than 1000 μg./ml. The resistance was transferred linked to (StTe). Strains with non-transferable ampicillin resistance had MICs of about 128 μg./ml.

Since July 1966 all strains resistant to streptomycin or tetracycline and a large number of those sensitive to these antibiotics but resistant to sulphonamides or ampicillin have been tested for their ability to transfer their resistances to K12.

The results are shown in Table 3. On the whole the observations made in the preliminary study on selected strains were found to be generally applicable.

Table 3. *Resistance and resistance transfer of unselected strains of* Shigella sonnei

Quarter	No. of strains tested	% of all strains: Resistant to Su	Resistant to St	St MIC > 800 μg./ml.	Transferring St Resistance	Resistant to Te	Resistant to Amp*
1966/3	129	88	58	35	40	48	70 (7)
1966/4	174	60	48	32	16	19	91 (8)
1967/1	106	90	66	54	12	15	85 (8)
1967/2	187	92	64	60	4	5	92 (2)
1967/3	66	97	68	50	17	10	95 (8)

* Figures in brackets = % of all strains tested transferring Amp resistance.

Sulphonamides. Of the 550 strains resistant to sulphonamides, 528 (96 %) were tested for the ability to transfer this resistance to K 12. No transfer could be demonstrated with 8 of these strains.

The increased proportion of strains sensitive to sulphonamide in the last quarter of 1966 is accounted for by a large local outbreak in Southwark caused by a strain of *Sh. sonnei* resistant only to ampicillin.

Streptomycin. It is apparent that the high level, chromosomal streptomycin resistance has become more common in this area, whereas the proportion of strains with transferable resistance fluctuates. Only three of the 97 strains with low level (MIC 64–128 μg./ml.) resistance to streptomycin failed to transfer this resistance to K 12. In the third quarter of 1966 a strain with unusual characteristics caused a local outbreak of Sonne dysentery in Islington. This strain was resistant to Su, St, Te and Amp and transferred Su and (StTe). The streptomycin MIC was more than 800 μg./ml. and it was therefore an exception to the general rule that high level St resistance does not transfer. However, when the (StTe) resistant K 12 progeny were mated with a sensitive strain of *Sh. sonnei* and streptomycin resistant *Sh. sonnei* progeny were examined, it was found that these were resistant only to 64 μg./ml. of streptomycin. It was concluded that the original strain contained two streptomycin resistance determinants, a non-moving chromosomal resistance and transferable streptomycin resistance linked to tetracycline resistance.

Tetracycline. All strains resistant to tetracycline transferred this resistance to K 12. The obvious correlation in Table 3 between the percentage of strains with transferable St resistance and those resistant to Te is accounted for by the fact that 78 % of strains resistant to Te had a combined (StTe) transferable resistance.

Ampicillin. Five hundred and five strains resistant to ampicillin were tested. This represents 88 % of all strains resistant to Amp. Most of the strains not tested were those from the outbreak in Southwark referred to previously, from which strains with non-transferable resistance to ampicillin only were isolated. Forty (8 %) of the strains tested were resistant to more than 500 μg./ml. of Amp and transferred this resistance. The remaining strains had an MIC of less than 500 μg./ml. and this resistance was not transferable. All of the moving Amp resistance was linked either to (StTe) or to St alone.

DISCUSSION

The trend towards increasing antibiotic resistance of *Sh. sonnei* in the London area described in our previous paper (Farrant & Tomlinson, 1966) has continued. In the second and third quarters of 1967 59 % of incidents were caused by strains resistant to sulphonamides, streptomycin and ampicillin. Almost all the ampicillin resistance and 88 % of the streptomycin resistance was not transferable and presumably chromosomal. If the strains which cause future outbreaks of Sonne dysentery in the area are derived from strains already endemic, the trend towards increasing resistance is likely to continue.

On the other hand resistance to tetracycline is not apparently increasing. All of the tetracycline resistance so far encountered has been transferable and therefore represents a less stable genetic character than mutational resistance. This is confirmed by our previous experience that multiply resistant strains of *Sh. sonnei* appeared to possess only limited powers of spreading and tended to die out in the population. But most of the tetracycline resistance which we encountered was combined with a transferable streptomycin resistance. If it is assumed that strains with transferable antibiotic resistance acquire this from other organisms present in the gut, the transfer process would be favoured by antibiotic therapy. If a patient infected with *Sh. sonnei* had in his gut a donor of combined (StTe) resistance, treatment with either antibiotic might result in the selection of a strain of *Sh. sonnei* resistant to both.

Further characterization of antibiotic resistance, i.e. the determination of the MIC and the ability to transfer, was of value in 'labelling' strains of *Sh. sonnei* in epidemiological studies. Strains from epidemiologically related incidents were found to have the same properties. This was particularly striking in a reception centre (not included in the analysis) where a strain transferring Su and (StTe Amp) was present for over one year. Forty-four strains with these characters were tested during this period. The particular pattern was so uncommon in the rest of the area that it was inconceivable that we were observing the repeated introduction of strains from outside.

Our method of selecting strains, i.e. examining the first strain isolated from an individual or family would enable us to detect transferable resistance only where this was a property of the infecting strain or was acquired early in the course of infection. The extent to which subsequent isolations from the individual or family have transferable resistances not present in the prototype strain is the subject of a separate study.

SUMMARY

The incidence of antibiotic resistant strains of *Sh. sonnei* isolated in the London area is described. Strains were tested for their ability to transfer resistance to *E. coli* K12

Most strains were resistant to sulphonamide and this resistance was transferable.

An increasing proportion of strains was resistant to 800 μg./ml. of streptomycin, but transferable streptomycin resistance was less common and often associated with tetracycline resistance.

The proportion of strains resistant to tetracycline fluctuated but this resistance was always transferred.

Ampicillin resistance was common; only those strains resistant to 500 μg./ml. transferred this resistance.

Resistance to other antibiotics was very rare.

We are grateful to Dr E. S. Anderson, who stimulated our interest in transferable drug resistance and made available to us his strain of *E. coli* K12.

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Further studies on the antibiotic resistance of *Shigella sonnei*

II. The acquisition of transferable antibiotic resistance *in vivo*

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INTRODUCTION

It has been shown (Davies, Farrant & Tomlinson, 1968) that the antibiotic resistance of some strains of *Shigella sonnei* isolated in the London area can be transferred to a suitable recipient strain of *Escherichia coli*. Our previous study was confined to the examination of one strain, i.e. the first strain isolated, from each family incident. Transferable resistance would have been detected only if it was present in the infecting strain or if a sensitive strain had acquired resistance early in the course of infection in the individual. Lewis (1967) has shown that, in an outbreak of infection with *Sh. flexneri* type 2*a* in a mental hospital ward, strains isolated from different individuals showed different antibiotic resistance patterns and much of this resistance was transferable to *E. coli* K 12. It seemed important to attempt to assess the extent to which strains isolated from individuals or families showed antibiotic resistance different from that of the infecting strain.

It is the policy of many of the Local Health Authorities in our area to collect specimens of faeces from known and suspected cases of Sonne dysentery and from their contacts both for diagnosis and for clearance. This provided an opportunity to examine all the *Sh. sonnei* strains from family incidents and to compare them with the first. It seemed reasonable to assume that, within a family, any strain with an antibiotic resistance pattern which differed from the index strain only by a single transferable resistance, or by several resistance determinants transferred as a single unit, represented acquired resistance rather than a separate source of infection.

Our previous experience suggested that:

(*a*) Most strains of *Sh. sonnei* would be resistant to sulphonamide and that this resistance would be transferable to *E. coli* K 12.

(*b*) Many strains would be resistant to 800 μg./ml. of streptomycin and that this resistance would not transfer.

(*c*) Some strains might possess a low level transferable resistance to streptomycin.

(*d*) Tetracycline resistance would be relatively uncommon but would be transferable.

(*e*) Most strains would be resistant to ampicillin but only those resistant to 500 μg./ml. or more would transfer this resistance.

(*f*) That resistance to other antibiotics would be extremely uncommon.

It was decided therefore to examine all the strains isolated from family outbreaks for the following properties:

(*a*) Sensitivity to sulphonamides, streptomycin, tetracycline and ampicillin.

(*b*) Degree of resistance to streptomycin (800 μ/ml.) and ampicillin (500 μ/ml.).

(*c*) Ability to transfer resistance to streptomycin, tetracycline and ampicillin.

(*d*) Colicine type.

MATERIALS AND METHODS

The isolation and identification of *Sh. sonnei*, antibiotic sensitivity testing by the disk diffusion method and the technique for colicine typing have been described previously (Farrant & Tomlinson, 1966). We continued to use these methods for the first strain isolated (index strain) from each family incident.

In order to examine further strains from the index case and from other members of the family it was necessary to mechanise the techniques of sensitivity testing, colicine typing and testing for the transfer of antibiotic resistance. Specimens of faeces were plated on deoxycholate citrate agar direct and from selenite broth in the usual way. A typical colony, which agglutinated with *Sh. sonnei* antiserum on a slide, was picked onto a segment of a MacConkey plate. After overnight incubation each strain was subcultured onto a nutrient agar slope and stored until a suitable number of strains to be tested had accumulated. (In practice about 50–100 strains were tested once a week.)

A bacteriophage typing machine (Lidwell pattern) was adapted with a special head carrying twelve stainless steel $\frac{1}{16}$ in. rods set at $\frac{3}{4}$ in. centres. The rods were arranged in four rows (2-4-4-2) so that they could be used to inoculate a $3\frac{1}{2}$ in. Petri dish. Nutrient broth was dispensed in round-bottomed $3 \times \frac{1}{2}$ in. agglutination tubes set in racks at the same centres and arrangement as the rods. A lid was fitted over each set of twelve broths.

The rods were sterilized by immersion in boiling water and were first used to mark nutrient agar templates. Strains of *Sh. sonnei* to be tested were then inoculated over the marks on the templates and incubated for 4 hr. An extra template was inoculated with *E. coli* K12 F^-Met^- (K12) over each mark. Growth was then transferred from the templates to the broths using the rods. Two sets of broths were inoculated from each template of *Sh. sonnei* strains, one set to determine antibiotic sensitivity and the other, with K12, to determine antibiotic resistance transfer. At the same time two plates of colicine medium were inoculated from each *Sh. sonnei* template. The broths were incubated overnight at 35° C. and the colicine plates at 33° C.

Antibiotic sensitivity tests

Plates of media containing suitable concentrations of antibiotics were inoculated from the overnight broth cultures using the rods. All plates were then incubated overnight at 35° C.

The concentrations of antibiotics were chosen after a number of strains, whose minimal inhibitory concentration (MIC) had previously been determined, had been examined using the rods to deliver the inoculum onto plates containing various concentrations of antibiotic. It was possible to select, for each antibiotic, a concentration at which all strains previously classified as 'sensitive' would be completely inhibited but on which all 'resistant' strains would grow. The concentrations used were:

Sulphathiazole, 10 μg./ml.
Streptomycin sulphate, 10 μg./ml.
Terramycin hydrochloride, 5 μg./ml.
Ampicillin, 5 μg./ml.

An additional plate containing 500 μg./ml. of ampicillin was used to distinguish the highly resistant strains.

The medium used for these sensitivity tests was that described by Davies (1954) except that it contained 0·14 % basic fuchsin. This dye was added merely to distinguish the plates from the minimal agar containing antibiotics used for transfer experiments and referred to later.

This medium was used for three reasons:

(*a*) It gave clear-cut results in sulphonamide sensitivity tests even with the relatively large inoculum deposited by the multiple inoculator.

(*b*) It permitted the recognition of strains of *Sh. sonnei* exacting for amino acids. These strains are infrequent but when they occur this character is a useful epidemiological 'label'. The sensitivity of such strains was determined by the disk method.

(*c*) It did not support the growth of K12 which is exacting for methionine. It was possible therefore to determine the antibiotic sensitivity of *Sh. sonnei* strains from the mixed broths although in practice a set of broths containing the *Sh. sonnei* strains alone was used for this purpose.

The test for high level resistance to streptomycin was performed on nutrient agar containing 800 μg./ml. of streptomycin sulphate.

The results were read after overnight incubation at 35° C. as growth or no growth on the appropriate medium.

Colicine typing

The colicine plates which had been incubated overnight at 33° C. were exposed to chloroform and the growth was removed with a sterile slide. One plate of each set was inoculated with indicator strain 17 (Abbott & Shannon, 1958) and the other with indicator strain Row, using a sterile swab to spread the inoculum. The plates were then incubated overnight at 35° C. Where a strain of *Sh. sonnei* had produced colicine a circular area of inhibition of growth (approx. 1·5 cm. diameter) was seen with one or both indicator strains. With the indicator strains used, four results were possible:

No inhibition	Colicine type 0
Inhibition of strain 17 only	Colicine type 7
Inhibition of strain Row only	Colicine type 6 or 12
Inhibition of both strains	Colicine type other than 0, 7 6 or 12

Since about 80 % of the strains of *Sh. sonnei* isolated from the index cases were colicine type 0 or 7 the use of these two indicator strains was appropriate to check the identity of subsequent isolations from the same family incident. When the index strain was of some other colicine type, inhibition of both indicator strains was accepted as evidence of identity unless some change in antibiotic sensitivity had occurred. Then the colicine typing was repeated by the standard technique using nine indicator strains.

Resistance transfer

The multiple inoculator was used to transfer the inoculum from sets of broths containing a mixed growth of *Sh. sonnei* and K12 to plates of minimal agar containing antibiotics. The minimal agar, described previously (Davies *et al.* 1968) would support the growth of K12 but was nutritionally deficient for *Sh. sonnei*. It was found that at the appropriate concentration of antibiotic (streptomycin 10 μg./ml., terramycin 10 μg./ml., ampicillin 25 μg./ml.) growth would occur on the minimal medium only if antibiotic resistance had been transferred to K12. A control plate of minimal agar containing no antibiotics was included since some strains of *Sh. sonnei* produced enough colicine in the mixtures to inhibit K12 so that transfer could not be demonstrated by this screening technique. When no growth occurred on the control plate or when resistance to more than one antibiotic was transferred the test was repeated. A loopful of the mixed culture was plated on minimal medium containing antibiotics so that the drug resistance pattern of single colonies could be investigated. The criteria for deciding whether resistance determinants were transferred as a single unit or independently are given in our previous paper (Davies *et al.* 1968).

RESULTS

Between March and September 1967 952 strains of *Sh. sonnei* were examined. These represented the first strains isolated from 221 index cases and all subsequent strains from the index case and his family contacts. The index strain from 199 incidents had no transferable antibiotic resistance other than to sulphonamide; the remaining 22 index strains transferred resistance to streptomycin, tetracycline or ampicillin.

Changes in antibiotic resistance in the index case

Sh. sonnei was isolated a second time from 127 of the 199 index cases originally excreting a strain with no transferable resistance other than to sulphonamide. Nine of these second isolates differed from the index strain. From these 127 patients 151 further strains were examined, and those from two differed from the previously isolated strains. The details are shown in Table 1.

It will be seen that all strains showing a change in antibiotic resistance could transfer the newly acquired resistance determinant to K12. In three incidents (nos. 3, 4 and 7) there was a change not only of antibiotic resistance but also of colicine type. In each case the ability to produce the specific colicine was also transferable and was linked to the new resistance determinant.

Twenty-three further strains were examined from fourteen index cases whose first strain transferred resistance to streptomycin, tetracycline or ampicillin. No changes in resistance pattern were observed.

It might be supposed that the acquisition of new resistance would be more common in those index cases whose first isolate was multiply resistant. This was not the case; 126 index strains were resistant to sulphonamide, streptomycin and ampicillin; the strains from four patients acquired further resistances (nos. 5, 6, 7 and 11). The forty-nine patients whose index strains were resistant to sulphonamide and ampicillin or to sulphonamide and streptomycin produced four 'new' resistances while strains from three of the twenty-four patients whose index strains were resistant only to sulphonamide or to ampicillin acquired resistance.

Table 1. *Changes in antibiotic resistance in strains of* Shigella sonnei *isolated from index cases*

	Index strain		Subsequent strains			
Incident no.	Resistance pattern	Colicine type	Resistance pattern	Transferable resistance*	Colicine type	Strain showing change†
1	SuAmp	0	SuStTeAmp§	Su, (StTeAmp)	0	2nd (5)
2	SuAmp	0	SuStTeAmp	Su, (StTe)	0	2nd (20)
3	SuAmp	0	SuTeAmp	Su, Te	2	2nd (9)
4	SuAmp	0	SuTeAmp	Su, Te	2	2nd (6)
5	SuSt‡Amp	0	SuSt‡Amp§	Su, (StAmp)	0	2nd (7)
6	SuSt‡Amp	0	SuSt‡TeAmp	Su, (StTe)	0	3rd (12)
7	SuSt‡Amp	0	SuSt‡TeAmp	Su, (StTe)	4	2nd (6)
8	Amp	1B	TeAmp	Te	1B	2nd (9)
9	Amp	1B	StTeAmp	(StTe)	1B	4th (37)
10	Amp	4	StTeAmp	(StTe)	4	2nd (8)
11	SuSt‡Amp	4	SuSt‡TeAmp	Su, Te	4	2nd (12)

Su = sulphonamide, St = streptomycin, Te = tetracycline, Amp = ampicillin.

* Parentheses, in this column, indicate resistances transferred as a single unit.

† Parentheses, in this column, indicate number of days after isolation of the index strain.

‡ Resistant to 800 μg./ml. streptomycin.

§ Resistant to 500 μg./ml. ampicillin.

Changes in antibiotic resistance in other members of the family

Members of the families of 165 index cases were found to be infected. A total of 651 strains from 350 infected family contacts were examined. Of these, 319 persons (605 strains) came from 148 families in which the index strain did not transfer resistance other than resistance to sulphonamide. From eighteen of these patients strains with 'new' resistances were isolated. The families of index cases whose strains initially possessed transferable resistance to streptomycin, tetracycline or ampicillin showed no changes in resistance pattern.

The details of the eighteen strains with 'new' resistances are shown in Table 2.

The ten patients from the first five incidents belonged to families in which strains from the index case also showed an altered resistance pattern. Sometimes the same change was observed in several members of the family (nos. 1, 7 and 11) but in other families members excreted organisms of different patterns (nos. 4 and

5). In some families it could be argued that change had occurred in one individual who then infected others with the altered strain. This is possible in incident no. 1 where either the index case or patient '*a*' could have infected patient '*b*' with the altered strain. In incident no. 5, however, patients '*a*', '*b*' and '*c*' were all infected with the index strain since the changes were not observed until the second or subsequent strain from these patients.

All members of the family were not necessarily involved when a change was observed. In incidents nos. 5, 7, 14 and 16 some individuals continued to excrete organisms with the characteristics of the index strain.

As with the index cases all newly acquired characters were transferable and where multiple characters were acquired these were transferred as a single unit.

Table 2. *Changes in antibiotic resistance in strains of* Shigella sonnei *isolated from family contacts*

	Index strain		Strains from members of family			
Incident no.	Resistance pattern	Colicine type	Resistance pattern	Transferable resistance*	Colicine type	Strain showing change†
1	SuAmp	0	(*a*) SuSt‡TeAmp§	Su, (StTeAmp)	0	1st (0)
			(*b*) SuSt‡TeAmp§	Su, (StTeAmp)	0	1st (5)
4	SuAmp	0	SuSt‡TeAmp§	Su, (StTeAmp)	0	2nd (12)
5	SuSt‡Amp	0	(*a*) SuSt‡Amp§	Su, (StAmp)	0	2nd (9)
			(*b*) SuSt‡Amp§	Su, (StAmp)	0	4th (21)
			(*c*) SuSt‡TeAmp	Su, (StTe)	4	4th (22
7	SuSt‡Amp	0	(*a*) SuSt‡TeAmp	Su, (StTe)	4	1st (2)
			(*b*) SuSt‡TeAmp	Su, (StTe)	4	2nd (8)
11	SuSt‡Amp	4	(*a*) SuSt‡TeAmp	Su, Te	4	1st (3)
			(*b*) SuSt‡TeAmp	Su, Te	4	1st (11)
12	SuSt‡Amp	0	SuSt‡TeAmp	Su, (StTe)	0	2nd (7)
13	SuSt‡Amp	0	SuSt‡TeAmp	Su, Te	0	1st (5)
14	SuSt‡Amp	0	(*a*) SuSt‡TeAmp	Su, (StTe)	0	1st (0)
			(*b*) SuSt‡TeAmp§	Su, (StTeAmp)	0	3rd (7)
15	SuAmp	7	SuStTeAmp	Su, (StTe)	7	1st (7)
16	SuSt‡Amp	7	SuSt‡Amp	Su, St	7	1st (13)
17	Amp	4	(*a*) SuAmp	Su	4	1st (8)
			(*b*) SuAmp	Su	4	1st (14)

Su = sulphonamide; St = streptomycin; Te = tetracycline; Amp = ampicillin.
* Parentheses in this column indicate resistances transferred as a single unit.
† Parentheses in this column indicate number of days after isolation of the index strain.
‡ Resistant to 800 μg./ml. streptomycin; § Resistant to 500 μg./ml. ampicillin.

DISCUSSION

The examination of strains of *Sh. sonnei* from convalescent patients and their family contacts showed some to be excreting strains with antibiotic resistance patterns different from those of the strains with which they were initially infected. The acquisition of new characters was not, however, a very frequent event, occurring in about 5% of infected persons (11 out of 199 index cases and 18 out of 319 contacts) or in 17 out of 199 incidents (8·5%).

These calculations are based on those incidents in which the index strain transferred no resistance other than that to sulphonamide, and undoubtedly underestimate the real frequency of acquired resistance. Where the index strain possessed transferable resistance this could indicate initial infection with a strain with this character, or it could mean that resistance had been acquired by a relatively sensitive strain before the first isolation was made. There were twenty-two families in which the index strain possessed transferable resistance to streptomycin, tetracycline or ampicillin. Four of these families were epidemiologically related, the index strain being isolated from the same borough in the same week and having the same characters and three other pairs of families were similarly related, so that sixteen epidemiological incidents were caused by these strains. These sixteen incidents were widely distributed in time and space, appearing as isolated examples of a strain with these characters in the boroughs concerned. However, in eleven instances there had been isolated from the same borough at the same time a possible 'parent' strain, i.e. a strain with a resistance pattern and colicine type lacking only the transferable resistance of the relevant index strain. In one case the evidence was strong, for the index strain had transferable resistance to sulphonamide and tetracycline and non-transferable resistance to ampicillin, it was colicine type 0 and exacting for tryptophan. Four other incidents had occurred in the borough during the previous two months from which identical strains—apart from the tetracycline resistance—had been isolated. Tryptophan-dependence is a very unusual character of *Sh. sonnei*, these five incidents being the only examples which were observed in this series. It seems unlikely that the tryptophan-dependent tetracycline-resistant strain was unrelated to the tetracycline-sensitive strains.

If these incidents are included in the calculation then of a total of 215 incidents there was evidence for acquired, transferable antibiotic resistance in 28 families (13%).

The effect which transferable antibiotic resistance has on the proportion of strains of *Sh. sonnei* in the population resistant to various antibiotics will be determined by a number of factors. The most important of these may be the stage of infection at which the resistance is acquired since this, among other things, will determine the probability of infection with the new resistant strain being passed on to start a local epidemic. A good example of what may happen was observed in two day nurseries (not included in the family outbreaks described here).

At the beginning of November a strain of *Sh. sonnei* colicine type 9 (uncommon in this area), resistant to sulphonamide, streptomycin and ampicillin, was isolated from a number of cases in the borough of Camden. During the first 3 weeks of November a total of twenty-two persons was found to be infected with this strain including nine children attending Day Nursery 'A' and four of their home contacts. On 22 November nine children attending Day Nursery 'B' were found to be excreting *Sh. sonnei*. The strains from four of these children were identical with that known to be present in the borough, viz. colicine type 9, sensitive to tetracycline, but the other five children were excreting a strain of the same colicine type but resistant to tetracycline. Up to the end of the month a total of twenty-one children attending Day Nursery 'B' and fifteen of their family contacts were found to be infected, seven with the tetracycline-sensitive strain and twenty-nine with the tetracycline-resistant strain. Up to the end of the year eight further persons in the borough with no obvious connection with Day Nursery 'B' were infected with *Sh. sonnei* colicine type 9. In four of these cases the strain was resistant to tetracycline. We do not know whether the acquisition of tetracycline resistance occurred only once or whether it occurred in a number of individuals. In either case the strain with the new character, arising at the beginning of an epidemiological incident, spread to produce a local outbreak.

The strains examined in this investigation were single-colony picks from specimens shown to contain *Sh. sonnei*. It seemed possible that many more persons were excreting organisms with acquired characters than were detected by this technique. Unless such organisms predominated we would have been unlikely to pick them. To estimate the extent of this source of error, 117 specimens of faeces which had been shown by the standard technique to contain tetracycline-sensitive strains of *Sh. sonnei* were plated on deoxycholate citrate agar containing 10 μg./ml. terramycin hydrochloride. From two patients a single colony of *Sh. sonnei* resistant to tetracycline was isolated. In neither case was *Sh. sonnei* isolated on a subsequent occasion so it was not possible to determine whether the resistant strain could have become predominant. On the other hand the 117 specimens contained two from individuals from whom a subsequent strain showed acquired tetracycline resistance. Plating on tetracycline-containing medium did not enable us to anticipate this change.

No information was sought on the antibiotic treatment, if any, that individual patients received. The presence of antibiotics in the gut might well favour the survival and continued multiplication of organisms with appropriate acquired resistance. But strains from patients infected initially with multiply resistant strains appeared no more likely to acquire new resistance than those from patients whose initial strain was relatively sensitive. The chance of *Sh. sonnei* acquiring resistance would also depend upon the presence in the gut of a suitable donor. Further studies on the incidence of coliform organisms with transferable antibiotic resistance in the faeces of patients infected with *Sh. sonnei* are in progress.

SUMMARY

A method is described for the testing of strains of *Sh. sonnei* for antibiotic resistance, colicine type and drug resistance transfer using a multiple inoculator.

The examination of 731 strains of *Sh. sonnei* isolated from convalescent cases and their family contacts showed that about 5 % of individuals were excreting organisms which differed from the infecting strain by a single transferable antibiotic resistance determinant. It is suggested that the acquisition of such resistance may occur in 13 % of family incidents.

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