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Salmonella javiana infection in an infant associated with a marsupial, the quokka, *Setonix brachyurus*, in Western Australia

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SUMMARY

Salmonella javiana, a serotype rarely isolated in Australia, has been recovered from the faeces of a 14-month-old infant with symptoms of enteritis.

The child had been closely associated with a marsupial species, the quokka, during a vacation on Rottnest Island in Western Australia, and *S. javiana* was isolated from faecal pellets from adult quokkas, and also from a snake collected on the island.

Sampling revealed a high incidence of *Salmonella* infection in the quokkas. In all, 62 out of 87 animals (71 %) were found to be infected, and 17 *Salmonella* and 3 *Arizona* serotypes were identified from 100 isolations comprising 92 salmonellas and 8 arizonas. Multiple infections were frequently detected and up to four serotypes were recovered from individual animals. *S. javiana* was isolated from four quokkas.

A close parallel was observed between the serotypes isolated from quokkas and sea-gulls on the island, and abattoir effluents, lake waters, bird droppings and reptiles sampled on the adjacent mainland.

The epidemiological significance of *Salmonella* and *Arizona* infections in the quokka population and their possible association with the seasonal decline in condition and numbers of animals on Rottnest Island is discussed.

INTRODUCTION

Salmonella javiana was first isolated from the faeces of a child affected with gastro-enteritis on the island of Java, and from two symptomless carriers in Panama (Edwards & Bruner, 1942). In the same year the organism was responsible for a serious outbreak of infection in a group of Navajo Indians in New Mexico (Alley & Pijoan, 1942). A total of 15 patients were admitted to hospital and there were two deaths. The source of infection was traced to a locally prepared cottage cheese.

Over the years the majority of *S. javiana* infections in humans have occurred in

the United States of America, particularly in southern states bordering the Gulf of Mexico (Edwards, Bruner & Moran, 1948; Sanders *et al.* 1965; Martin & Ewing, 1969). Occasional human isolations have been reported from Canada and several European, Asian and South American countries (Kelterborn, 1967). *S. javiana* has also been isolated from symptomless children in rural areas of Ceylon (Gulasekharam & Velaudapillai, 1961) and from West Malaysian aborigines (Anandan, Lim & Haug, 1969). A single family outbreak has been reported from Australia (Atkinson, Carter, Wollaston & Wall, 1953). The serotype was isolated from three children with gastro-enteritis and from two healthy carriers.

In contrast to human isolations, only a few *S. javiana* strains have been recovered from animal sources; however, the serotype has been isolated from poultry, pigs, dogs and cats in America (Edwards *et al.* 1948; Bruner & Moran, 1949; Galton, Smith, McElrath & Hardy, 1954), a Canadian mink (Bynoe & Yurack, 1964), livestock in Holland (Kelterborn, 1967), and pigs in England and the Philippines (Taylor, 1969; Arambulo, Westerlund & Sarmiento, 1968). *S. javiana* has also been isolated from Chinese egg, desiccated coconut from Ceylon and Indian bone products, as well as human sources in Ceylon, England, Malaya, New Zealand and British Guiana (Semple, Graham & Dutton, 1961; Taylor *et al.* 1965).

The present case was diagnosed in a 14-month-old infant who had developed enteritis during a family holiday on Rottnest Island, a popular tourist resort 20 km. from the mainland close to Perth. *S. javiana* was isolated on two occasions from the child's faeces. In attempts to trace the source of the infection, it was noted from laboratory records that a single culture identified as a monophasic *S. javiana* strain had been isolated previously from a snake (*Demansia nuchalis affinis*) captured on the same island. There was no history of direct contact with reptiles; however, it was established that during the holiday period, and before the onset of symptoms, the child had been closely associated with the local quokka (*Setonix brachyurus*), a small marsupial wallaby present in large numbers on the island, and had frequently been observed playing with faecal pellets scattered throughout the settlement area.

A sampling programme was therefore initiated, and *Salmonella* investigations were undertaken, concentrating firstly upon faecal pellets scattered on the ground throughout the settlement area. The survey was then extended to include rectal swabs and faecal samples collected from adult quokkas from all parts of the island. The animals were swabbed immediately following capture and then held in cages until fresh faeces were voided. Finally, in an attempt to identify possible vectors for the *Salmonella*, many of which were serotypes commonly found in abattoir effluents from the mainland, cloacal samples were collected from sea-gulls (*Larus novaehollandiae*).

MATERIALS AND METHODS

Faecal samples from the infected infant and contacts of the case were collected and examined after the family had returned to the mainland. Animal droppings were collected on the island close to the holiday accommodation and throughout the settlement area.

Rectal swabs, previously moistened with buffered glycerol saline faeces transport medium (Sachs, 1939), were collected in duplicate from 87 quokkas and 83 silver gulls captured close to the settlement and from more remote areas of the island. Faecal samples were also examined from individual animals and birds held captive overnight. Samples of sea water were tested using Moore swabs immersed in the nearby harbour for 24 hr.

Faeces and swab samples were plated out direct on Oxoid deoxycholate-citrate (D.C.) agar and modified bismuth sulphite (B.S.) agar, and inoculated into strontium chloride B enrichment broth (Iveson, 1971) incubated at 43° C. and strontium selenite A broth (Iveson, 1973) incubated at 37° C. The enrichment media were plated out at 24 and 48 hr. intervals on D.C. and modified B.S. agar, and colonies were identified biochemically and serologically.

RESULTS

Direct culture of the child's faeces revealed a heavy growth of *S. javiana*. No other serotypes were detected by multiple-isolation techniques. *S. javiana* was again isolated a week later, but was not detected in subsequent faecal samples examined after 2 weeks, 6 weeks and 3 months.

Faecal samples from other family members, the domestic cat and persons who had shared or visited the holiday accommodation were all negative. The staff and children from a kindergarten attended by the elder sister were also examined with negative results. Foodstuffs consumed on the island were not available for examination; however, other children and adults who had attended the various meals had remained free of symptoms both before and during the vacation period. Moore swabs immersed in the settlement harbour were also negative.

Examination of quokka droppings collected throughout the settlement area showed that 50% (13) of the samples were infected. *S. anatum*, *S. chester* and *S. havana* were isolated close to the hut previously occupied by the family, and *S. javiana*, *S. decatur*, *S. muenchen*, *S. newington*, *S. typhimurium* and *Arizona* 26:26:25 were isolated from samples collected in the surrounding area. Multiple infections were detected in four samples and one sample yielded three serotypes.

A total of 100 isolations comprising 92 *Salmonella* and 8 *Arizona* species were recorded from 62 out of 87 adult quokkas (71%); 17 *Salmonella* and 3 *Arizona* serotypes were identified. Multiple infections were again frequently encountered and up to four serotypes were detected in individual animals. *S. javiana* was isolated from four individual animals. The distribution and relative frequency of *Salmonella* and *Arizona* serotypes in samples collected from individual quokkas, sea-gulls and their droppings are detailed in Table 1.

DISCUSSION

Salmonella organisms have been previously isolated from both healthy and diseased marsupials, and in some cases have been associated with fatal infections in captive animals (Atkinson *et al.* 1953; Lee & Mackerras, 1955; Winter & O'Connor, 1957; Yadav, Stanley & Waring, 1972). *Salmonella* infection in wild

Table 1. Salmonella *and* Arizona *serotypes from Rottnest Island and adjacent mainland sources*

	Rottnest Island			Mainland			
Serotype	Quokkas	Quokka droppings	Seagulls	Seagull droppings	Reptiles	Lake water	Abattoir effluents
S. adelaide	2	.	1	2	3	2	14
S. anatum	.	1	.	2	1	4	10
S. bootle	1	.	.	.	6	.	.
S. newbrunswick	1	.	.	.	.	.	.
S. bahrenfeld	9	.	.	.	2	.	5
S. chester	1	3	.	.	4	.	5
S. decatur	.	1	.	.	1	1	.
S. derby	.	.	1	8	.	14	44
*S. fremantle**	2	.	.	.	4	.	.
S. give	1	.	.	17	5	.	2
S. havana	1	6	.	2	1	7	24
S. javiana	4	2	.	.	.	.	.
S. muenchen	23	1	.	.	6	3	2
S. newington	17	2	.	2	1	2	9
S. oranienburg	3	.	.	.	6	2	5
S. orientalis	4	.	.	.	11	.	1
S. orion	5	.	.	1	.	.	3
S. typhimurium	8	1	2	4	9	5	13
*S. wandsbek**	9	.	.	.	8	.	3
S. waycross	1	.	.	.	1	.	.
A. 16:23:25	2	.	.	.	12	.	.
A. 26: 23:21	1	.	.	.	2	.	.
A. 26:26:25	5	1	.	.	23	.	.
E. tarda	.	.	.	.	6	10	.
Total isolations	100	18	4	38	112	50	140

* Salmonella subgenus II.

marsupials has not been previously reported from Australia; however, samples of kangaroo meat sold as pet food have been shown to be contaminated with many serotypes including *Arizona* species (Laurie & Kovacs, 1960; Anderson, Crowder & Woodruff, 1964; Vernon, 1966; Iveson & Mackay-Scollay, 1972).

Infections of marsupials with pathogenic Enterobacteriaceae have not been confined to Australian animals, and *Salmonella* and *Arizona* bacteria have been isolated from marsupials in the United States of America (Edwards, McWhorter & Fife, 1956; Schnurrenburger *et al.* 1968; Marx, 1969) and from South America (Lins, 1970).

The results of the present investigation have not only implicated the quokkas as the most likely source of *S. javiana* infection in the infant, but have also drawn attention to the need for further studies to evaluate the epidemiological significance of *Salmonella* infections within the quokka community.

The quokka (*Setonix brachyurus* (Quoy & Gaimard)) is a small nocturnal marsupial weighing approximately 3 kg and with a ruminant-like digestion (Moir,

Somers & Waring, 1956). The wallaby was once widespread on the mainland of Western Australia, being gazetted as a pest in the 1930s, but following an undocumented catastrophe the species is now restricted to two small offshore islands, Rottnest Island and Bald Island.

The total quokka population on Rottnest Island has been estimated at 5000 individuals or one per acre (Waring, 1956) and during the summer months, which extend from November to March, the animals undergo a marked decline in condition characterized by a loss of body weight, dehydration, a fall in body temperature and severe anaemia. It is during this period that mortality within this population is highest, affecting younger animals more than adults (Shield, 1959).

Over the past 15 years the quokka has been the centre of a number of investigations aimed at documenting changes in population size and elucidating possible causes of mortality, but seasonal debility has not been attributable to any single nutritional or physiological factor (Main, Shield & Waring, 1959; Storr, 1964*a*; Main 1970). Furthermore, the only pathogen isolated previously from morbid animals has been the hookworm *Austrostrongylus thylogale*, which was also present in large numbers in freshwater soaks frequented by the animals during the summer months (Waring, 1956). It was suggested that, while hookworm would certainly contribute to anaemia and loss of condition, protein deficiency was probably the major factor associated with seasonal loss of condition, which might be exacerbated in areas of the island lacking free water (Storr, 1964*a*).

The results of the present investigation have revealed a widespread and unexpected reservoir of infection in the quokka and this raises the question of whether the quokka (and other marsupial species) are natural carriers of *Salmonella* and *Arizona* bacteria or whether the high infection rate is related to the adverse conditions of late summer and perhaps contributes to the seasonal debility of animals in the field. More intensive sampling from animals in better condition on a seasonal basis will be needed before this question can be answered.

Salmonella and *Arizona* isolations from mainland marsupials have been largely from captive animals and, in general, isolation rates have been lower than those from quokkas (see Table 2). However, *Salmonella* organisms have been recovered from the majority of species tested, and high rates of infection have been detected, particularly in animals subjected to stress associated with transportation; for example, 70% of droppings collected from a truck used to transport grey kangaroos (*Macropus fulginosus*) were infected with *Salmonella* or *Arizona* species. *S. jangwani* was also isolated from four short-eared wallabies (*Petrogale brachyotis*) with symptoms of acute enteritis, shortly after arrival in Perth from the Ord River area. On the other hand *Arizona* 26:26:25 was isolated from a healthy wild tammar (*Macropus eugenii*) immediately after capture on the Abrolhos islands.

The occurrence of anaemia in Rottnest quokkas has been observed to coincide with the decline of good-quality forage and with increased attendance at soaks, but was not associated in previous investigations with a raised leucocyte count or rise in the rate of sedimentation (Shield, 1958, 1959). Barret-Connor (1972) and Weinburg (1971) have recently reviewed the known associations of infection and anaemia, including haematological aspects of *Salmonella* infection and the role of

Table 2. Salmonella *and* Arizona *isolations from captive marsupials in Western Australia*

Species	Tests: Animals	Tests: Droppings	Total positive	Isolations
Macropus robustus (Euro)	18	—	2	*S. typhimurium* (1) *S. decatur* (1)
M. fulginosus (Grey kangaroo)	—	10	7	*S. adelaide* (1) *S. muenchen* (2) A. 26:26:25 (4)
M. eugenii (Tammar)	35	—	2	*S. newington* (1) A. 26:26:25 (1)
Megaleia rufa (Red kangaroo)	1	—	1	*S. wandsbek* (1)
Petrogale brachyotis (Short-eared wallaby)	4	—	4	*S. jangwani* (4)
Dasyurus geoffroii (Native cat)	4	—	4	*S. charity* (2) *S. chester* (1) *S. muenchen* (2)
Sminthopsis murina (Marsupial mouse)	1	—	1	*S. fremantle* (1)
Isoodon obesulus (Bandicoot)	1	—	1	*S. meleagridis* (1)
Tachyglossus aculeatus (Echidna)	1	—	1	*S. anatum* (1)
Totals	65	10	23	24

plasma iron in host–parasite interactions. Further studies are clearly required to assess the role of *Salmonella* infection in the quokka population, as the combination of a low-grade diet, protein-deficiency anaemia, dehydration, hookworm and *Salmonella* infection and pregnancy could be expected to reduce the viability of an animal population restricted to a small island, and collectively operate to the detriment of the host species.

The importance of the carrier animal as a source of *Salmonella* outbreaks in livestock, and the possible relation of infection to stress factors associated with inadequate diet and overstocking, has been reported by Josland (1953). It was also observed that the carrier state could be maintained for long periods in animals restricted to an infected environment. Enrichment culture was the only reliable method for the detection of carriers.

The significance of stress and poor physical condition on the incidence of salmonellas in animal populations harbouring a small number of carriers has been emphasized by Salisbury (1958).

Adverse factors which have influenced rates of *Salmonella* infection in wild animal populations have also been reported by Taylor (1968), who suggested that, while *Salmonella* were present in the healthy host in non-epidemic periods, adverse conditions might precipitate an active infection in one or several animals, thus initiating an outbreak. Contamination of the animal's environment would then be

much greater than that caused by the healthy carrier. Outbreaks of *S. typhimurium* infection in birds had been noted towards the end of adverse seasons when there was a scarcity of suitable food.

The multiplication of *Salmonella* organisms in the rumen of cattle, followed by intestinal infection and excretion of the organisms in faeces, have also been closely related to variations in the food intake (Brownlie & Grau, 1967).

Several factors point to the mainland as being a likely and possibly recent source for at least some of the *Salmonella* strains isolated from the adult quokkas. *S. adelaide*, *S. derby* and *S. typhimurium* were isolated from sea-gulls on the island and have been frequently detected in mainland abattoir effluents and nearby lakes on the mainland frequented by sea-gulls. Furthermore, 13 *Salmonella* serotypes isolated from the quokkas, which together made up 90% of the isolations, have also been recovered from a single mainland abattoir complex which discharges effluent into sea water only 25 km. from Rottnest Island. *S. adelaide*, *S. anatum*, *S. derby*, *S. give*, *S. havana*, *S. newington*, *S. orion* and *S. typhimurium* have also been isolated from bird droppings collected at a lakeside landfill site situated in close proximity to the abattoir complex.

Excretion of several *Salmonella* serotypes by wild gulls has been recorded previously by Windle Taylor (1967). Carriage of *Salmonella* by birds has been regarded as a transient feature, but the possible build-up of infection when large numbers of birds congregate together has been documented by Mitchell & Ridgwell (1971). Apart from humans and sea-gulls a further possible vector for *Salmonella* is wild ducks, which periodically migrate to Rottnest Island to breed, but sampling of 240 mainland black ducks (*Anas superciliosa*) yielded only five *E. tarda* strains and one isolation of *Salmonella wandsbek* – two species rarely associated with either urban or agricultural activity.

Apart from the quokkas, there are no other native mammals on the island; however, at varying intervals over the past 100 years, cattle, sheep, horses, dogs, cats, rabbits, foxes, rodents and several avian species, including peacocks and pheasants, were introduced. These have been restricted in more recent times to pheasants, peacocks, domestic mice, a few feral cats, and a small group of horses available to tourists. Animal feedingstuffs and organic fertilizers have also been used particularly in areas close to the settlement. The island is visited by several migratory bird species including the mutton bird (*Puffinus pacificus*), which nests on the island; however, predatory birds are few and lizards and snakes are abundant (Dunnet, 1962). Silver gulls are at all times plentiful and there is a regular interchange of birds between the island and mainland, particularly in the breeding season (Storr, 1964*b*).

The isolation of *S. javiana* from a snake, an infected child and from the faeces of four adult quokkas has drawn attention to the potential role of reptiles in the interchange of *Salmonella* and *Arizona* serotypes on the island. Reptilian species have been shown to be important natural reservoirs of infection in many parts of the world. *S. javiana*, for example, has been isolated from wild reptiles in Vietnam (Le Minor, 1964) and the Philippines (Westerlund, 1966), and from a captive snake in Germany (Schroder, 1970). Both captive and free-ranging reptiles in Western

Australia have been found to be frequently infected with *Salmonella*, *Arizona* and *Edwardsiella* organisms (Iveson, Mackay-Scollay & Bamford, 1969; Iveson, 1971).

With the exception of only *S. newbrunswick*, *S. javiana* and *S. orion* the serotypes recovered from quokkas have also been isolated from reptiles captured within 35 km. of Rottnest on coastal areas of the adjacent mainland. The importance of geographical isolation in speciation and subsequent adaptation and differentiation of organisms is well known, and although the isolation of the Rottnest fauna occurred before the more recent advent of European man and his domestic animals in the Australian region, it is not surprising, in view of the short distance involved, that there is a close relation between the serotypes occurring in the quokka population and strains recovered from the nearby mainland. Furthermore, the distribution patterns of serotypes will be derived from both the earlier presence on the mainland of reptilian and marsupial host species and, in more recent times, the introduction of organisms resulting from ecological changes brought about by the development of agriculture, domestication of animals and intensifying human settlement, on both the island and adjacent coast.

It is evident that epidemiological studies alone do not explain the extreme diversity of *Salmonella* and *Arizona* serotypes, and high rates of infection in the small colony of quokkas. Furthermore, it is surprising, in view of the large numbers of serotypes involved, that *S. javiana*, which was recovered from only four (4·6%) animals, was the only strain associated with the human infection. Further seasonal studies of host–parasite relationships in selected mainland and island populations of native and introduced animals are required, to establish more precisely the origins and distribution patterns of individual serotypes, and their role as potential pathogens in the Rottnest quokka population.

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The sequential appearance of antibody and immunoglobins in nasal secretion after immunization of volunteers with live and inactivated influenza B virus vaccines

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SUMMARY

The sequential development of the immune response in nasal washings was studied in 54 volunteers immunized with either attenuated or inactivated influenza B/Eng/13/65 virus vaccines.

Eleven of the 15 volunteers given the inactivated vaccine by deep subcutaneous inoculation showed no rise in nasal wash protein or immunoglobins due to the immunization procedure nor was specific neutralizing antibody detected in their nasal washings after immunization. Neutralizing antibody was detected in nasal washings of three volunteers in this group who also showed a 20-fold or greater increase in serum haemagglutinin-inhibiting antibody after immunization and in one volunteer who had antibody present in pre-trial nasal washings.

Eleven of 15 volunteers who were successfully infected by the live attenuated vaccine showed a characteristic rise in protein and IgA and IgG immunoglobin concentrations in nasal washings 5–14 days after the administration of the live virus vaccine. Neutralizing antibody was detected in the nasal washings of these 11 volunteers and appeared at the same time as or 1–2 days after the initial rise of protein and immunoglobin. Neutralizing antibody was also detected in the nasal washings of one other volunteer who did not show a rise in protein or immunoglobin concentration in nasal washings after immunization.

IgA was detected ($\geqslant$ 3 mg./100 ml.) in the majority (84 %) of nasal wash specimens which had a protein concentration of 0·2 mg./ml. or greater while IgG was not detected ($\geqslant$ 4·5 mg./100 ml.) until the protein concentration rose to 0·4 mg./ml. or greater. The geometric mean concentration for normal nasal wash protein in this study was $0{\cdot}3 \pm 0{\cdot}1$ mg./ml.

Regression analysis indicated that the concentrations of both IgA and IgG immunoglobins were directly proportional to the protein concentration in nasal washings but that this relationship varied considerably between individuals.

Absorption studies indicated that neutralizing and haemagglutinin-inhibiting antibodies in nasal secretion to influenza B/Eng/13/65 virus were predominantly associated with the IgA class of immunoglobin.

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INTRODUCTION

Early studies (Burnet, Lush & Jackson, 1939; Francis, 1941; Fazekas de St Groth & Donelley, 1950) indicated that neutralizing antibody to influenza virus in respiratory secretions might play an important part in resistance to further infection.

Artenstein, Bellanti & Buescher (1964), Remington, Vosti, Lietze & Zimmerman (1964), Rossen, Schade, Butler & Kasel (1966) and Alford, Rossen, Butler & Kasel (1967), have since confirmed that neutralizing activity found in nasal secretion after respiratory virus infections was due to specific antibody and belonged to a different immunoglobin class (IgA) than that found in serum (IgG).

IgA (11S) has been found to be the predominant immunoglobin in nasal secretion and is generally thought to be synthesized locally (Tomasi, Tan, Solomon & Prendergast, 1965; South *et al.* 1966; Butler, Rossen & Waldman, 1967; Rossen *et al.* 1968). However, the presence of IgG in nasal secretion has not been well documented and until recently (Butler *et al.* 1970) there has been little published information on the sequential appearance of these two immunoglobins in relation to nasal secretion antibody after infection or immunization by respiratory viruses.

The present study investigated the sequential development of the immune response in nasal secretion with particular attention to the production of IgG and IgA immunoglobins after three immunization procedures using live and inactivated influenza B virus vaccines.

MATERIALS AND METHODS

Virus vaccines

A live attenuated influenza B/Eng/13/65 virus vaccine (Beare, Bynoe & Tyrrell, 1968) was kindly supplied by Dr D. C. Breeze, Evans Medical Ltd., Speke. This virus had a titre of $10^{9\cdot4}$ EID 50/ml. Formalin-inactivated vaccines containing $5\cdot5 \times 10^3$ haemagglutinating units (HAU)/ml. were prepared by Evans Medical Ltd. from an influenza A/Eng/1/61 (H2N2) virus strain and from the same strain of influenza B virus as the live attenuated vaccine.

Volunteers

The design of the vaccine trials has been described previously (Downie & Stuart-Harris, 1970). A total of 54 medical students were immunized with influenza B virus vaccines during two vaccine trials in October 1967 and October 1968. In the 1967 trial, eight volunteers were given an intranasal dose of 1·0 ml. of the live attenuated influenza B virus vaccine diluted in Hanks's saline to contain $10^{5\cdot4}$ EID 50/ml. of virus and a second group of seven volunteers was inoculated by deep subcutaneous injection with 1·0 ml. of the inactivated influenza B virus vaccine. In the 1968 trial 11 students were given 1·0 ml. of either the live influenza B virus vaccine ($10^{5\cdot4}$ EID 50/ml.) or 1·0 ml. of the inactivated influenza B virus vaccine intranasally, a further eight were given 1·0 ml. of the same inactivated vaccine subcutaneously and a control group of nine students was given 1·0 ml. of the influenza A2/Eng/1/61 virus vaccine subcutaneously. One month after the

initial immunization, all the volunteers were given an intranasal dose of 1·0 ml. of live influenza B/Eng/13/65 virus vaccine diluted to contain $10^{6\cdot4}$ EID 50/ml. of virus. The first inoculation of virus in the 1968 vaccine trial was designated as the immunizing dose and the second as the challenge dose of virus.

Serial nasal washings were collected as described previously (Downie & Stuart-Harris, 1970) before and after immunization and after challenge during a total period of 21 days in 1967 and 53 days in 1968. The nasal washings were tested for the presence of haemoglobin by the guaiac test and positive specimens were discarded. Serum specimens were collected before immunization, 3 weeks after immunization and 3 weeks after challenge and stored at $-20°$ C.

Haemagglutination-inhibition (HI) test

Assays of haemagglutinin (HA) and the inhibition of haemagglutination (HI) by serum and nasal washings were done according to the method described by Fazekas de St Groth & Webster (1966). Before testing, all nasal washings were treated with receptor-destroying enzyme (RDE). A normal rabbit serum, a normal nasal washing containing 2 mg./ml. of protein and a specific rabbit antiserum of known titre to influenza B/Eng/13/65 virus were included as controls.

Neuraminidase-inhibition (NI) test

Assay of neuraminidase-inhibiting (NI) antibody was done as described previously (Downie, 1970).

Neutralization test

Titrations of neutralizing antibody in nasal washings were done according to the Quantitative Haemadsorption (QH) test described by Finter (1967). A specific rabbit antiserum of known titre was included in each test and the nasal washings were heated at 56° C. for 30 min. before testing.

Complement fixation (CF) test

CF tests were carried out by standard methods using two exact units of complement, and overnight fixation at 4° C.

Assay of immunoglobins

The immunoglobins in nasal washings were assayed by the radial diffusion precipitation method in agar (Mancini, Carbonava & Heremans, 1965). A stabilized human reference serum (Hoechst Pharmacueticals Ltd., London) was used to measure IgA, IgG and IgM immunoglobin levels in nasal washings. Since the rate of diffusion in agar is dependent upon molecular size, the values obtained for nasal washing IgA (11S) will be falsely low in comparison to serum IgA (7S) and therefore will not be absolute values for this immunoglobin. However, comparison between different nasal wash specimens will still be valid.

IgA and IgG were specifically absorbed from nasal washings with an equal volume of anti-human immunoglobin serum at optimal precipitating concentrations as previously described (Downie, 1970). Goat anti-human IgA serum was

obtained from Hyland Laboratories, Los Angeles, California, and goat anti-human IgG and IgM sera were kindly supplied by Professor P. G. H. Gell, Department of Experimental Pathology, Birmingham University, England.

Protein assay

Protein concentrations of nasal washings were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard.

RESULTS

Relation of protein and immunoglobins in nasal washings

Protein estimations were done on every nasal washing collected from all volunteers during 1967 and 1968. The observations for 1967 and 1968 showed that the geometric mean concentration for normal nasal wash protein for the volunteers was $0{\cdot}3 \pm 0{\cdot}1$ mg./ml. and concentrations of greater than 1·0 mg./ml. were considered to be abnormal.

Quantitative assay for IgA, IgG and IgM were done on every nasal washing collected from all volunteers during 1967 and from those volunteers in 1968 who showed a fourfold rise in serum antibody after immunization or challenge or who showed a threefold rise in nasal wash protein concentration. IgM was detected in 14 of the 1100 nasal wash specimens assayed for immunoglobins. All 14 specimens were collected during a rise in protein either due to the live influenza B virus vaccine or an unrelated common cold-like infection and the mean concentration of protein in these specimens was 5·2 mg./ml.

IgA was generally found in measurable amounts ($\geqslant$ 3mg./100 ml.) when the concentration of nasal wash protein was 0·2 mg./ml. or greater, and IgG was generally measurable ($\geqslant$ 4·5 mg./100 ml.) when the protein concentration was 0·4 mg./ml. or greater. Since the protein concentration of normal wash was 0·3 mg./ml., the majority (84 %) of specimens contained measurable concentrations of IgA (3–6 mg./100 ml.) but not IgG.

The relation between protein concentration and the concentrations of IgA and IgG in nasal washings was examined using grouped data regression analysis on results obtained from 27 individuals totalling 347 observations for IgA and 248 for IgG (Figs. 1, 2). A group consisted of all observations on IgA or IgG at a particular concentration of protein. A linear relationship best fitted to the values for both immunoglobins was calculated by the method of least squares (Dixon & Massey, 1957) and the slopes of these lines were significantly different from zero ($P < 0{\cdot}01$). This suggested that both nasal secretion IgA and IgG were proportional to the concentration of nasal secretion protein. Thus, at protein concentrations of 1·0 mg./ml. or greater both IgA and IgG tended to have values of 10 mg./100 ml. or greater. However, both analyses showed significantly higher deviations from the line of regression than would be expected on the basis of observed variation within groups although the contribution of regression to variation in both IgA and IgG was still highly significant. Thus, the relation could not be regarded as a simple one. One possibility was that it was curvilinear, although similar analyses

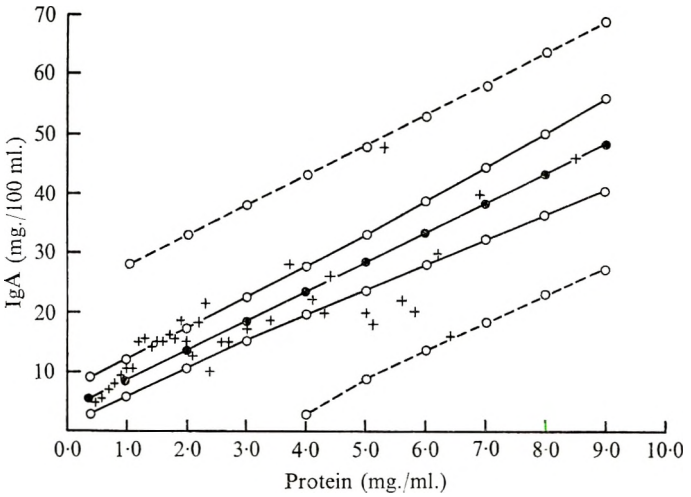

Fig. 1. Grouped data regression equation analysis of IgA immunoglobin concentration and nasal wash protein concentration (347 observations). ●—●, Line of best fit; ○—○, 90% limits (fiducial) on predicted value; ○--○, tolerance limits for predicting future single values.

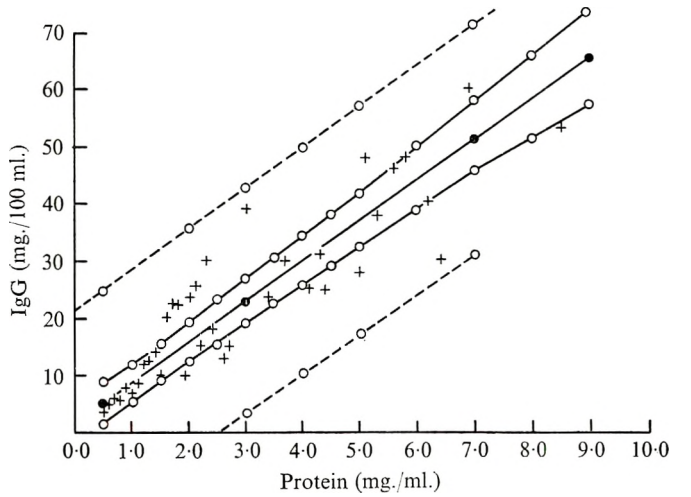

Fig. 2. Grouped data regression equation analysis of IgG immunoglobulin concentration and nasal wash protein concentration (248 observations). ●—●, Line of best fit; ○—○, 90% limits (fiducial) on predicted value; ○--○, tolerance limits for predicting future single values.

using $X = \log (10 \times \text{protein})$ still showed significant deviations from regression between groups. Another possibility was that different individuals were making a greater contribution to the variance between groups than to the within-group variance, i.e. that the relation of protein concentration to IgA and IgG concentrations varied from individual to individual. To test this possibility the same analysis was carried out separately on four individuals and it was found that the majority of the deviations from regression due to the variance between groups almost entirely disappeared. Thus, the relation between IgA and IgG and protein con-

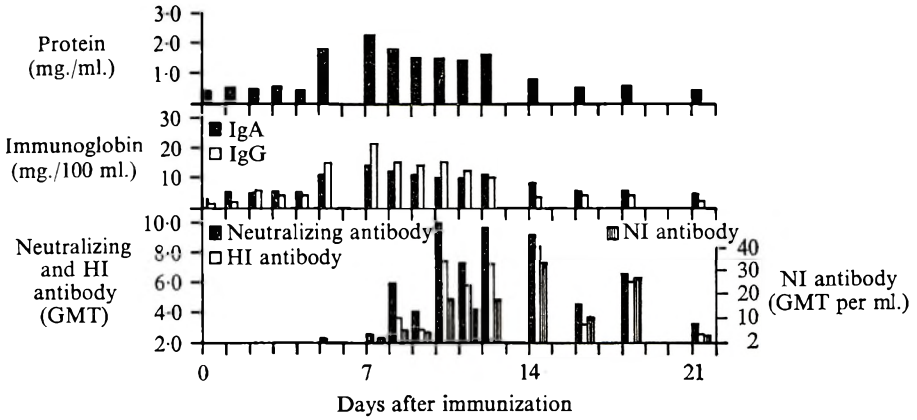

Fig. 3. Sequential changes in protein, immunoglobin and antibody concentrations in the nasal washings of 15 volunteers showing serological evidence of infection after either the immunization or challenge dose of live attenuated influenza B virus vaccine. Neutralizing HI and NI antibody are expressed as the reciprocal of the geometric mean titres (GMT). Protein and immunoglobin concentrations are expressed as the arithmetic mean.

centrations was highly significant ($> 0{\cdot}1\,\%$) for each individual. Joint regression analyses were performed to test the hypothesis of individual difference and the best fitting lines describing the relation of protein concentration with the concentration of IgA and IgG differed significantly in slope between individuals. Thus, much of the variation between groups due to deviations from regression must have stemmed from these individual differences.

Immune response in nasal washings after immunization

The sequential development of the immune response in nasal secretion to immunization with live and inactivated influenza B/Eng/13/65 virus vaccines was studied by assaying serial nasal washings from each volunteer for protein, immunoglobins (IgA, IgG and IgM), and specific virus neutralizing, NI, and HI antibodies. Antibody titres were adjusted to values per ml. and expressed as the reciprocal of the geometric mean titres (GMT). Protein and immunoglobin concentrations were expressed as the arithmetic mean.

Immune response in nasal washings – live virus vaccine given intranasally

Neutralizing, HI and NI antibodies were detected in the nasal washings of 12 out of 15 volunteers who showed serological evidence of infection after either the immunization or challenge dose of live influenza B virus vaccine. These 15 volunteers included 6 from the 1967 trial and 1 from the 1968 trial who were immunized with the live virus vaccine, and 8 from the 1968 trial who were challenged with the live virus vaccine.

Eleven of the 12 volunteers with nasal-wash antibody also showed a characteristic increase in the concentration of nasal wash protein and immunoglobins to levels of from three to five times greater than that found before immunization. The sequential changes in nasal-wash protein, immunoglobins and antibody con-

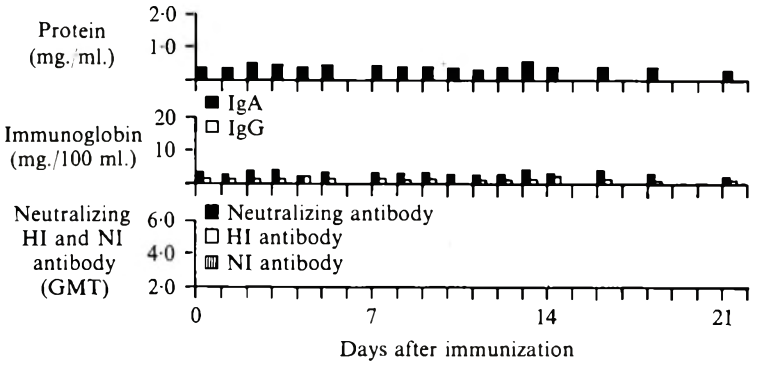

Fig. 4. Sequential changes in protein, immunoglobin and antibody concentrations in the nasal washings of 11 volunteers successfully immunized with inactivated influenza B virus vaccine by deep subcutaneous injection. Neutralizing, HI and NI antibody are expressed as the reciprocal of the geometric mean (GMT). Protein and immunoglobin concentrations are expressed as the arithmetic mean.

centrations of the 15 volunteers who responded serologically to the live virus vaccine are summarized in Fig. 3.

The rise in nasal-wash protein concentration (> 1·0 mg./ml.) and IgA and IgG immunoglobin concentrations (> 10 mg./100 ml.) was observed 5-14 days after the administration of live virus and lasted from 1 to 7 days in different individuals. IgG was generally detected only at the time of this increase in protein and reached maximum concentrations of 10-60 mg./100 ml. with a maximum mean of 21·6 mg./100 ml. while IgA concentrations (7S standard) ranged from 10 to 40 mg./100 ml. with a maximum mean of 15·4 mg./100 ml. Fourteen to 21 days after immunization the protein and immunoglobin concentrations returned to their normal values.

Neutralizing, HI and NI antibody appeared in the nasal washings 7–14 days after the administration of the live virus vaccine and could be detected up to 28 days.

Immune response in nasal washings – inactivated virus vaccine given subcutaneously

Eleven of the 15 volunteers given the inactivated influenza B/Eng/13/65 virus vaccine subcutaneously in the 1967 and 1968 trials showed no abnormal rise in nasal wash protein (> 1·0 mg./ml.) or immunoglobins (> 10 mg./ml.) due to the administration of vaccine, nor was neutralizing antibody to influenza B/Eng/13/65 virus detected in their nasal washings during the 21- to 28-day period after immunization. These results are summarized in Fig. 4 and are in sharp contrast to those shown in Fig. 3.

However, neutralizing antibody to influenza B/Eng/13/65 virus was detected in the nasal washings of the four remaining volunteers in this group; three showed a 20-fold or greater rise in serum HI antibody to the inactivated vaccine while the fourth had neutralizing antibody present in nasal washings before the administration of the vaccine.

Fig. 5 illustrates the pooled results for changes in nasal wash protein, IgA, IgG, and antibody concentrations of the three volunteers who had a 20-fold rise in serum HI antibody. Two of them were among six volunteers in the 1968 trial who

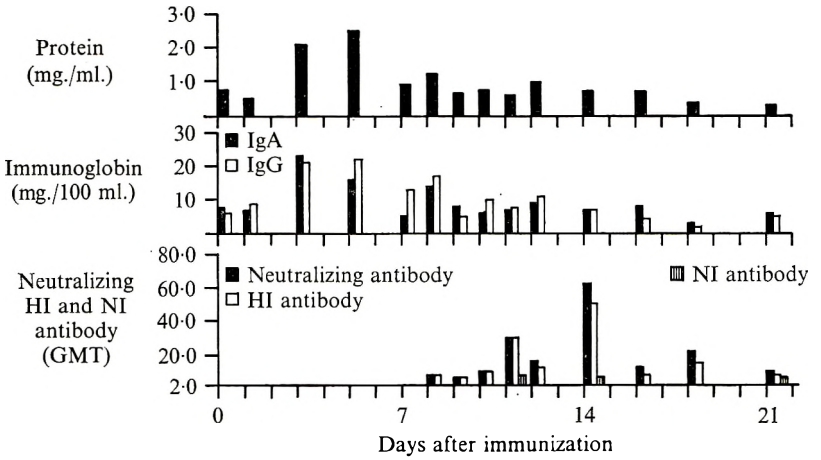

Fig. 5. Sequential changes in protein, immunoglobin and antibody concentrations in the nasal washings of three volunteers showing a 20-fold or greater rise in serum HI antibody after immunization with inactivated influenza B virus vaccine by deep subcutaneous injection. Neutralizing HI and NI antibody are expressed as the reciprocal of the geometric mean titres (GMT). Protein and immunoglobin concentrations are expressed as the arithmetic mean.

had a common cold-like illness unrelated to the immunization procedure during the post-immunization period which caused an increase in protein and immunoglobins 1–5 days after administration of the vaccine. In these two volunteers, nasal-wash protein, IgA and IgG concentrations were consistently higher than average ($> 0{\cdot}5$ mg./ml., and > 6 mg./100 ml., and > 5 mg./100 ml. respectively) throughout the trial period. Influenza B/65 virus neutralizing and HI antibodies were detected in their nasal washings in relatively high titres (GMT 1/30–1/60) from the 11th day after immunization to the end of the period of observation. NI antibody on the other hand was detected on only three occasions and in low concentration (GMT 1/7), although the serum titres for NI antibody were comparable with the titres in those who were infected by the live virus vaccine.

Immune response in nasal washings – inactivated virus vaccine given intranasally

Only one out of the 11 volunteers in the 1968 vaccine trial given inactivated influenza B virus vaccine intranasally responded serologically to the immunization procedure. There was no abnormal rise in nasal-wash proteins or immunoglobins due to the immunization procedure. Neutralizing antibody to influenza B/65 virus was detected in one specimen taken 8 days after immunization.

Relation of immunoglobins and antibody in nasal washings

IgA and IgG immunoglobins were specifically removed from nasal-wash specimens of volunteers immunized and challenged with influenza B/Eng/13/65 virus by precipitation with an equal volume of anti-human immunoglobin sera at optimal proportions. The absorbed washings were than tested for the presence of

Table 1. *Presence of neutralizing and HI antibody after specific absorption of IgA and IgG from nasal secretion of volunteers immunized and challenged with influenza B/Eng/13/65 virus* (1968)

Volunteer and immunization procedure	Day after Immunization*	Neutralizing antibody titre after absorption†				HI antibody titre after absorption†			
		Control	IgA absorbed	IgG absorbed	IgA–IgG absorbed	Control	IgA absorbed	IgG absorbed	IgA-IgG absorbed
Volunteer no. 6.	40	40	0‡	40	0	64	64	64	0
Live influenza B/Eng/13/65 intranasal	46	10	0	5	0	32	0§	16	0
Volunteer no. 31.	11	40	0	5	0	64	0	64	0
Inactivated influenza B/Eng/13/65 (subcutaneous)	12	80	0	20	0	64	0	64	0
	31	40	0	10	0	64	0	64	0
	44	30	0	20	0	64	0	32	0
Volunteer no. 35. Inactivated influenza A2/Eng/1/61 (subcutaneous)	42	10	0	5	0	16	0	16	0

* Volunteers were challenged on day 28.
† Reciprocal of titre.
‡ Neutralizing antibody titre < 5.
§ HI antibody titre < 8.

neutralizing antibody and, after treatment with RDE, for HI antibody. IgM immunoglobin was not detected in any of the specimens absorbed.

Table 1 gives the results from three volunteers in the 1968 trial for neutralizing and HI antibody in nasal washings after the absorption of IgA and IgG. In each instance removal of IgA immunoglobin also removed the neutralizing activity of the nasal washing, while removal of IgG had little or no effect on this activity. Absorption of IgA also removed the HI antibody from nasal secretions in all but one instance. This nasal washing was taken from volunteer no. 6, 12 days after the administration of the challenge dose of virus. Absorption of either IgA or IgG had no effect on the HI titre to influenza B virus, suggesting that specific antibody was associated with both immunoglobins. Removal of both immunoglobins also removed the HI antibody activity. A later specimen from the same volunteer, taken 18 days after challenge, showed that the HI activity was entirely in the IgA fraction. One possible explanation might be the transudation of serum IgG antibody into respiratory tract secretions. Therefore serum and nasal washings from volunteers no. 6, 31, 35 and three others who showed HI antibody rises in both serum and nasal washing to either immunization or challenge with influenza B/Eng/13/65 virus in 1968, were treated with RDE and tested for HI antibody to influenza A/Tokyo/3/66 (H2N2) virus. If transudation of serum antibody into nasal washings was primarily involved one might expect HI antibody to the A/Tokyo/3/66 virus in both serum and nasal washings.

All six volunteers had serum HI antibody titres greater than 1/24 to influenza A/Tokyo/3/66 virus but none of the nasal washings from the six had detectable amounts of HI antibody to this influenza A virus. These results suggest that transudation of antibody from the serum to the nasal secretion was probably not the primary source of antibody in the nasal washings.

DISCUSSION

The results of the present study have indicated that there were considerable differences in the sequential development of the immune response in nasal washings of the volunteers who received either live or inactivated influenza vaccines. The live influenza virus vaccine given intranasally was found to be more successful in stimulating neutralizing, HI and NI antibody in nasal washings than was the inactivated vaccine given either intranasally or subcutaneously.

In this study a characteristic rise in nasal-wash protein occurred 5–14 days after the administration of live virus and coincided with similar increases in the concentration of IgA and IgG immunoglobins. Neutralizing and HI antibodies reached maximum titres after 10–14 days and were generally still detected up to 28 days after the administration of the live virus vaccine when protein and immunoglobin concentrations had returned to pre-immunization levels.

These results are in agreement with previous studies (Rossen *et al.* 1970; Alford *et al.* 1967) in which neutralizing antibody appeared in nasal secretion between 7 and 28 days after administration of live influenza A virus, while the rise in nasal wash protein occurred 4–6 days after inoculation.

In contrast, inactivated influenza B virus vaccine given subcutaneously was

more efficient in stimulating serum antibody than antibody in nasal washings. The only volunteers in this group who developed detectable neutralizing antibody in nasal secretion after immunization also had a 20-fold or greater rise in serum HI antibody. Thus it seems probable that very high levels of circulating antibody need to be stimulated by subcutaneous vaccine before neutralizing or HI antibody can be detected in nasal secretion (Mostow *et al.* 1970). These results are consistent with the findings of other studies that commercially available inactivated vaccines given subcutaneously are poor inducers of nasal-secretion neutralizing antibody (Smith, Purcell, Bellanti & Chanock, 1966; Mann *et al.* 1968; Waldman, Mann & Kasel, 1968; Kasel *et al.* 1969; Fluk *et al.* 1970).

In the present study, considerable concentrations of IgG were found in nasal washings after the administration of live influenza B virus vaccine while in other studies (Rossen *et al.* 1965, 1966; Alford *et al.* 1967) only minimal amounts of IgG have been detected in nasal washings after influenza A virus infection. Moreover, in the present study, regression analysis indicated that the concentrations of both IgA and IgG were related to the concentration of both IgA and IgG were related to the concentration of protein in nasal wash and a rise in protein generally coincided with an increase in the concentration of both these immunoglobins. The relation between IgA and IgG and nasal wash protein was found to be highly significant when individuals were analysed separately ($< 0{\cdot}1\%$) but joint regression analysis indicated that this relation varied considerably between individuals.

Other findings suggest that there may be differences in the pattern of the local response in the host to different respiratory viruses. Butler *et al.* (1970) have shown that after infection by either rhinovirus type 15 or coxsackievirus A type 21, rises in the concentrations of IgA and nasal wash protein appeared to coincide while changes in IgG concentration appeared to follow those in nasal wash albumin. The peak concentration for IgG and albumin occurred 2–5 days earlier than that for IgA and nasal wash protein.

Specific absorption tests in this and in a previous report (Downie, 1970) have indicated that the neutralizing, HI and neuraminidase-inhibiting (NI) antibody activities in nasal secretion were predominantly associated with the IgA class of immunoglobin. These results confirm and extend those of other workers (Rossen *et al.* 1966; Alford *et al.* 1967; Mann *et al.* 1968) who have demonstrated that the neutralizing antibody to influenza virus in nasal secretion was predominantly associated with the 11 S IgA immunoglobin fraction.

Although substantial amounts of IgG were detected in nasal washing after the administration of live influenza B virus vaccine, it appeared to have little or no specific antibody activity. In only one instance in the present study did HI antibody to influenza B virus appear to be partly associated with IgG as well as with IgA immunoglobin. The role that this nasal-wash IgG may play in the immune response to respiratory infection is not known but earlier studies (Artenstein *et. al.* 1964; Bellanti, Artenstein & Buescher, 1965; Alford *et al.*, 1967) have indicated that part of the neutralizing antibody to influenza A virus in nasal secretion may be associated with 7S IgG immunoglobin.

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Electron-microscopic study of measles virus in lymphocytes of affected children

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SUMMARY

Two different types of inclusion bodies have been found by electron-microscopy in the cytoplasm of sectioned lymphocytes from children infected with measles.

The first is tubular, 18 nm. internal diameter, embedded in osmiophilic material, and is found during the incubation period. It is not thought to be specific for measles, and is probably identical with structures recently reported in HEp-2 cell lines and tumour tissue.

The second type is less obviously tubular and morphologically resembles measles nucleocapsid. This was found only after the rash had appeared.

INTRODUCTION

Although measles virus can be recovered with greater ease from the white cell fraction than from other elements of blood (Todd, 1928; Daubney, 1928; Smith, 1929; Douglas & Smith, 1930; Fenner & Woodroofe, 1953; Gresser & Chany, 1963) there appear to have been few electron-microscope studies of measles lymphocytes themselves. In the present work these cells have been examined early in the disease and the morphology of intracytoplasmic inclusions described.

MATERIALS AND METHODS

Blood from ten children was examined: 2 were incubating the disease (7 days before the rash appeared), 2 were in the first day, 4 were 2 days after the appearance of the rash, and 1 each at 14 and 17 days. For comparison, blood from ten normal children who had had measles within the last 2–5 years was also studied.

Lymphocytes were separated from about 5 ml of venous blood by the carbonyl iron and methyl cellulose method (Coulson & Chalmers, 1967; modified by Hughes & Caspary, 1970) and the cells in 199 solution were fixed for 1 hr. by addition of the same volume of double-strength (i.e. 8%) chilled buffered glutaraldehyde in 0·4 M sodium cacodylate solution at pH 7·2. The cells thus fixed in suspension were lightly centrifuged (250 *g* for 15 min.), the pellet post-fixed in 2% osmic acid and embedded in Epon. Sections were cut on an LKB 'Ultrotome', stained with lead citrate and uranyl acetate and examined in a Philips 200 or 300 electron microscope.

In one experiment lymphocytes which had been rapidly frozen in liquid nitrogen were thawed, and negative contrast staining carried out with 2% sodium phospho-

tungstate at pH 7·0. In another, fresh unfixed lymphocytes were disintegrated in a Griffiths tube and examined immediately by techniques as described by Narang, Bell & Gibson (1971).

RESULTS

In the incubation stage specimens many of the lymphocytes showed pseudopodial outgrowths and the cytoplasmic volume was increased and there were many ribosomes free from endoplasmic reticulum. Here and there were regularly arranged tubular elements sometimes cut in cross-section (Pl. 1, figs. 1, 2) and sometimes longitudinally (Pl. 2, figs. 3, 4). They were deeply stained and apparently embedded in osmiophilic material so that at first sight under low power they resembled ordinary dense bodies. The clear internal diameter of the individual tubular structures was 18 nm and their outer surface was often rather blurred where they were embedded in dense background material. Despite the superficial resemblance to dense bodies no relation either to these or to mitochondria was apparent.

On the second day of rash a second type of cytoplasmic inclusion characteristic of measles nucleocapsids was found in addition to those described above. Lightly staining rounded islands of cytoplasm, usually close to the nucleus, were seen. They were devoid of bounding membrane (Pl. 3, fig. 5). Sometimes more than one inclusion body was present in a single lymphocyte (Pl. 3, fig. 6, arrows). These islands comprised a network of tubular structures often with rather ill-defined boundaries, though here and there was evidence of a fine cross-striation (Pl. 4, figs. 7, 8). Sometimes most of the structures constituting an inclusion were cut transversely (Fig. 7) and their tubular nature was then clear. The diameter of the internal translucent area was 16 nm. The mass of tubules appeared embedded in an electron-dense 'fuzzy' matrix. Sometimes the inclusions were related to endoplasmic reticulum (Fig. 8) but no structural connexion was apparent.

Despite the large number of villous protrusions, no budding of virus particles from affected lymphocytes was seen. As a rule lymphocytes with inclusion bodies showed evidence of stimulation (e.g. increased endoplasmic reticulum and mitochondria) and often the large numbers of pseudopodial outgrowths associated with beginning degeneration of the cell.

No inclusions of the measles type shown in Figs. 5–8 were found in convalescent or uninfected subjects. However, the dark inclusion bodies (Figs. 1–4) seen in the incubation phase (continuing on into the early clinical stage) have also been seen in lymphocytes from four patients with leukaemia and one with carcinoma of ovary (Pl. 5, fig. 9) and cannot be regarded as specific for measles.

Negative contrast staining of frozen and thawed lymphocytes from a child on the second day of rash revealed nucleocapsids resembling those of measles virus (Pl. 5, fig. 10) though clear herring-bone formations were not discernible, but this may be because frozen-thawed material was used. The width of the structure was about 15 nm.

DISCUSSION

Although there is so much evidence that measles virus may grow in leucocytes, especially lymphocytes, virus has not been seen within these cells during clinical infection.

The patients from whom the lymphocytes were studied developed well-marked measles, with rash at the time of study or (when this was in the incubation stage) subsequently. Collateral study *in vitro* of the lymphocyte sensitization to measles antigens on the same patients showed this to be present in all cases (Field, Caspary, Shenton & Madgwick, 1973). Thus in one case macrophage electrophoretic slowing (Field & Caspary, 1971) rose from 2·4 % and 4·1 % for encephalitogenic factor and measles respectively, 7 days before onset of the rash, to 9·0 % and 12·2 % on the second day of the rash. This cellular sensitization preceded the appearance of humoral antibody demonstrated by complement fixation and haemagglutination inhibition tests on the same children (B. McLaughlin, H. Madgwick & E. J. Field, unpublished). In some cases blood was taken during the incubation stage and retaken during the rash or 17, 18 and 47 days after measles rash. Complement fixation went up from a titre of 1/2 on the 2nd day of rash to 1/512 on the 18th day after rash. On the other hand, haemagglutination inhibition was 1/32 on the 2nd day and went up to a titre of 1/256 on the 18th day in the same patient, thus showing the appearance of measles antibody. From these findings together with the absence of the structures seen in Figs. 5–8 in lymphocytes from other conditions it is probable that the structures described are limited to natural measles infection. This is further supported by the morphological resemblance of the inclusions to known measles nucleoprotein in the brain of mice infected by intracerebral inoculation with the Edmonston strain of measles virus (Figs. 11, 12) (E. J. Field, H. K. Narang and T. M. Bell, unpublished) and *in vitro* (Bell *et al.* 1972). Most lymphocytes which contained virus also showed evidence of stimulation and many were furnished with numerous villous projections – a feature commonly seen in degenerating cells. Dunn & Kernahan (1957) have postulated that the lymphopenia present in the early stages of certain virus infections may be due to destruction of affected cells and perhaps also their bone-marrow precursors.

The depression of delayed hypersensitivity reactions immediately after measles first described by von Pirquet in 1908 has since been several times confirmed. It may be that it is associated with viral colonization of lymphocytes.

Clarke, Attridge & Gay (1969) described tubular structures similar in morphology to the 1st type (Figs. 1–4) in HEp-2 cells infected with type 1 foamy agent. More recently, Berthiaume & Joncas (1973) in a study of the morphogenesis of respiratory syncytical virus have observed similar tubular structures, both in infected and control HEp-2 cells. The specificity of these structures is uncertain and their presence in lymphocytes from cancer and measles patients and HEp-2 cell line may suggest that they are occasional normal structures of unknown function, or abnormal structures resulting from disease or some other agent.

I am grateful to Professor E. J. Field for suggesting the problem and for his help and encouragement during the work; and to Miss Joyce Davison for technical assistance.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Lymphocytes from child on second day of rash. Note multiple pseudopodial villi and the density of ribosomes within the cytoplasm. The number of mitochondria is increased. The arrows show an accumulation of tubular structures mostly circular but some in longitudinal section (detail in Fig. 2). × 25,000.

Fig. 2. High-power view of inclusions in Fig. 1. Note groups of circular tubular structures of internal diameter 18 nm. × 154,000.

PLATE 2

Fig. 3. Lymphocytes from child about 4 days before appearance of rash. Note more densely staining inclusions which may be taken at first sight for dense bodies (arrows) (detail in Fig. 4). × 20,000.

Fig. 4. High-power view of inclusions in Fig. 3. × 82,000.

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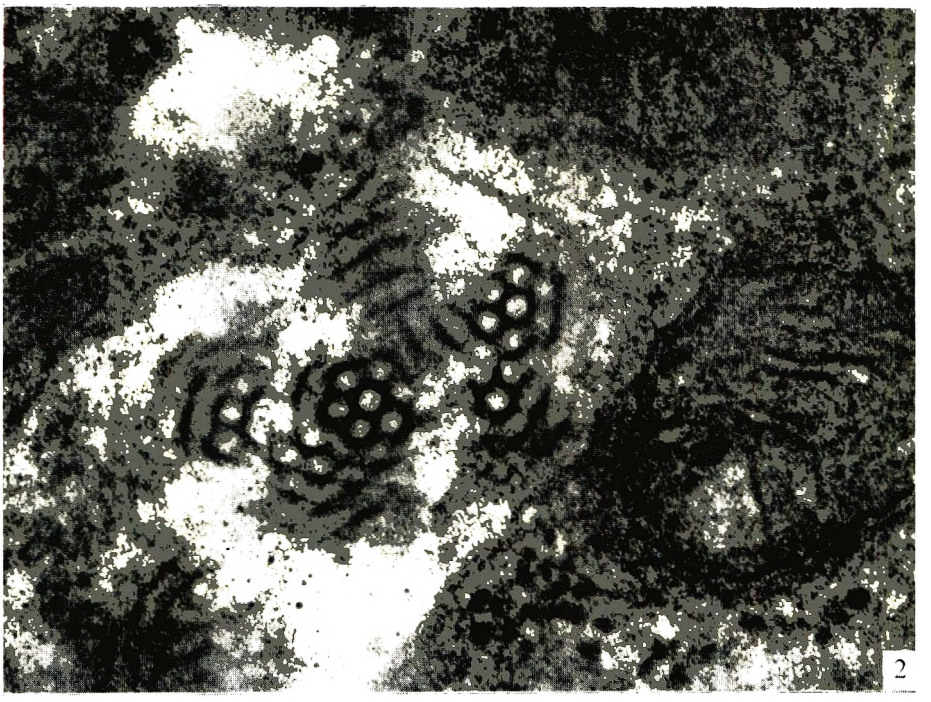

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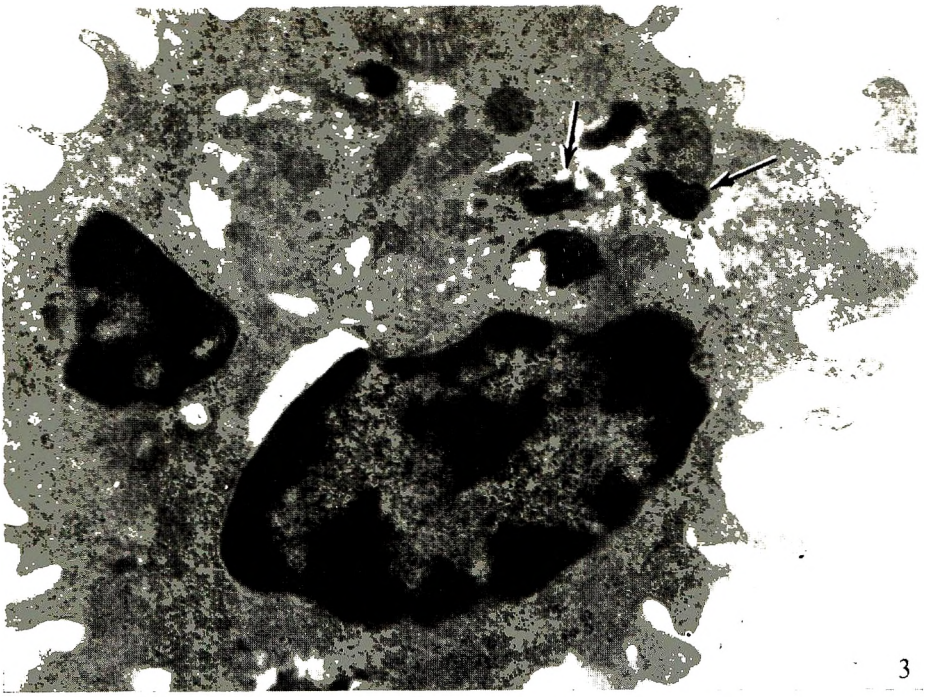
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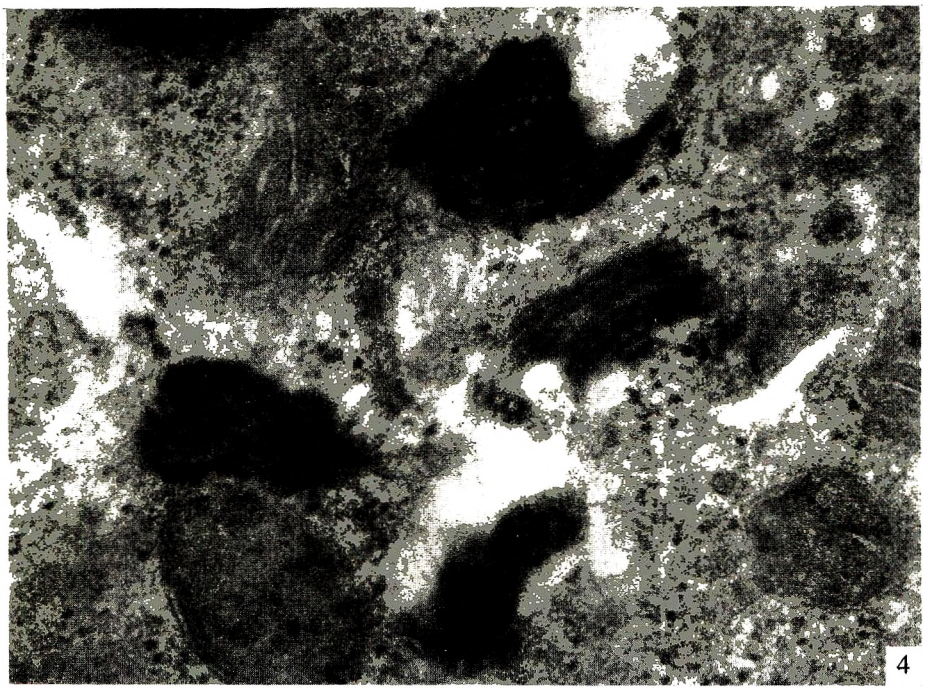
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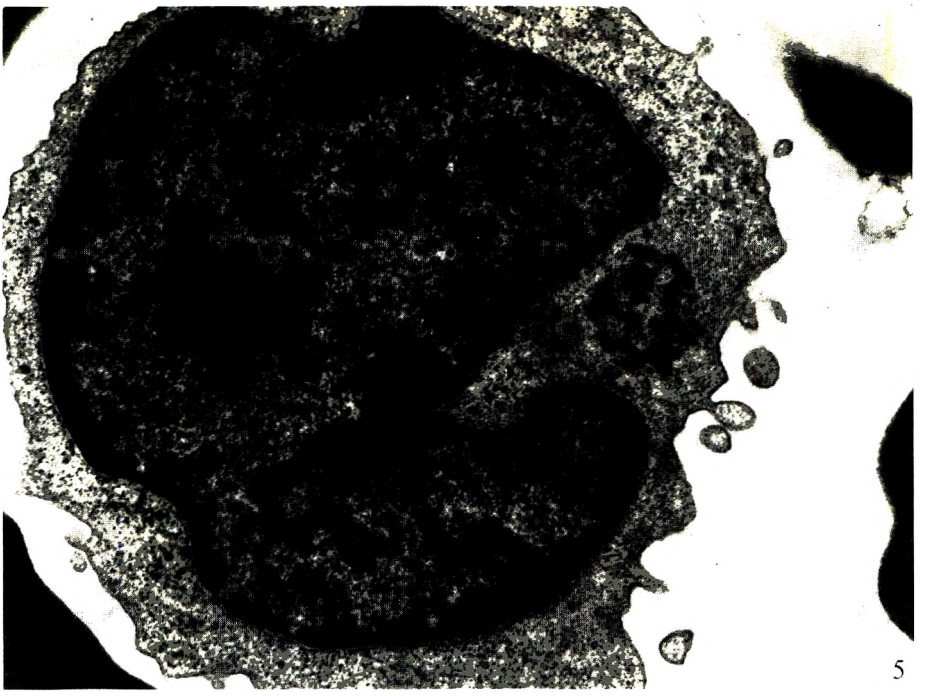

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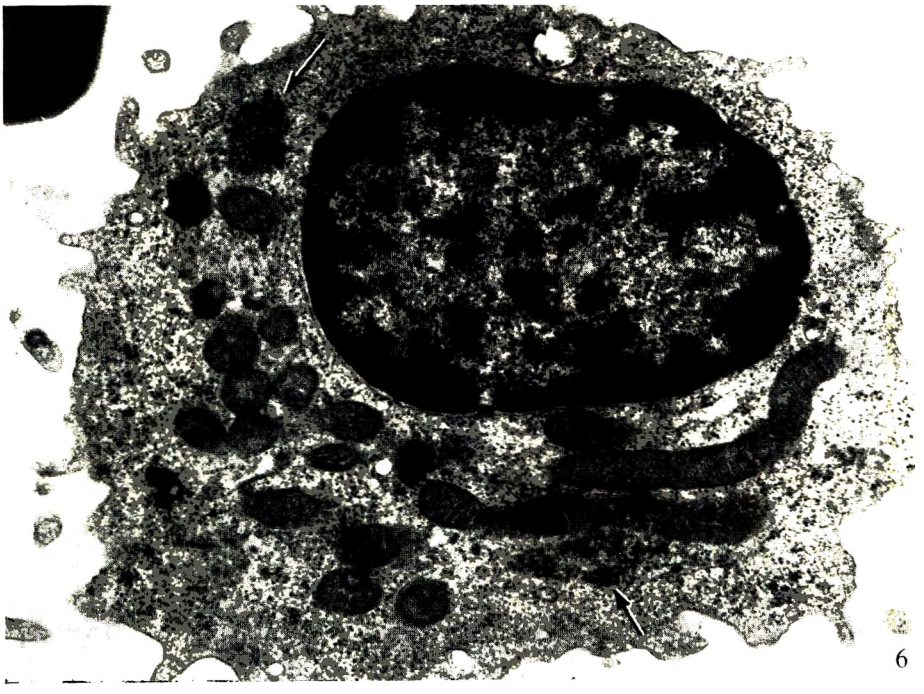

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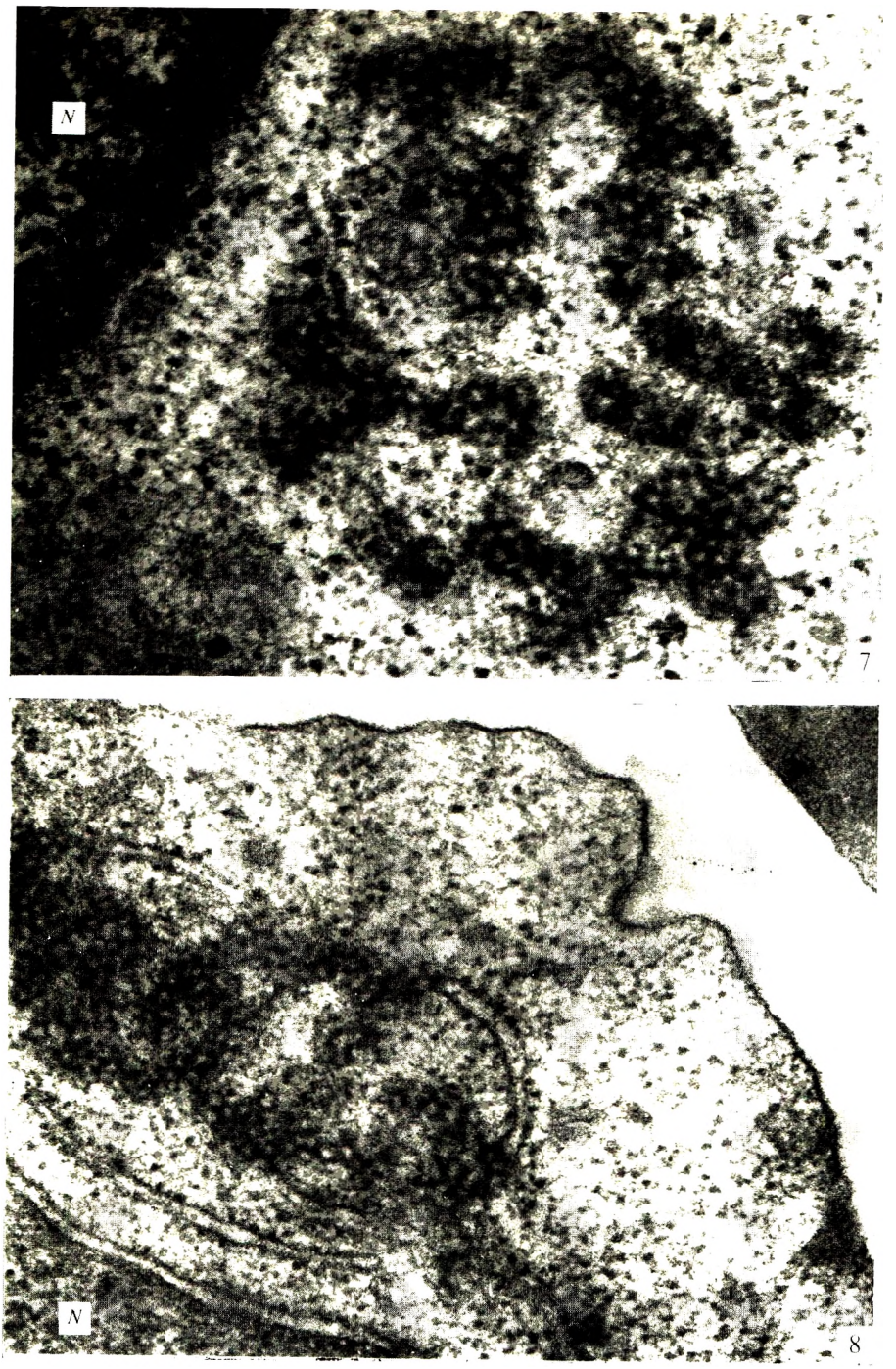
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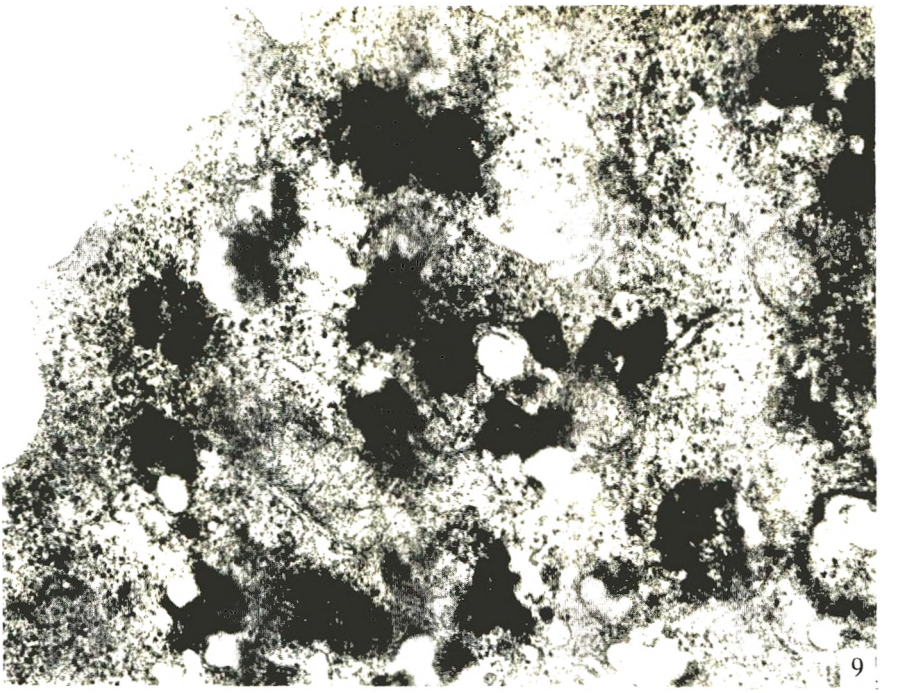
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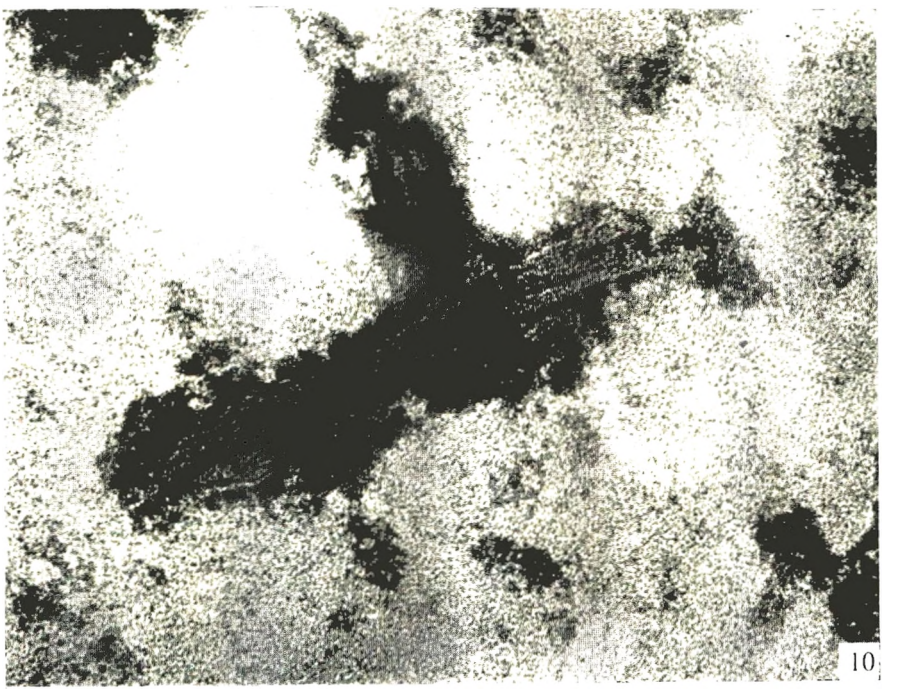
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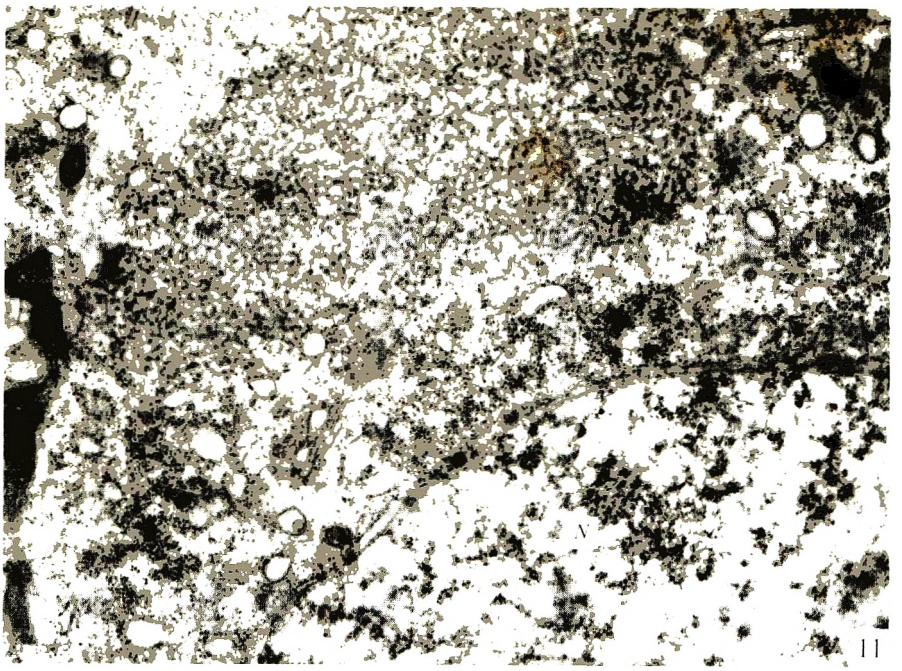
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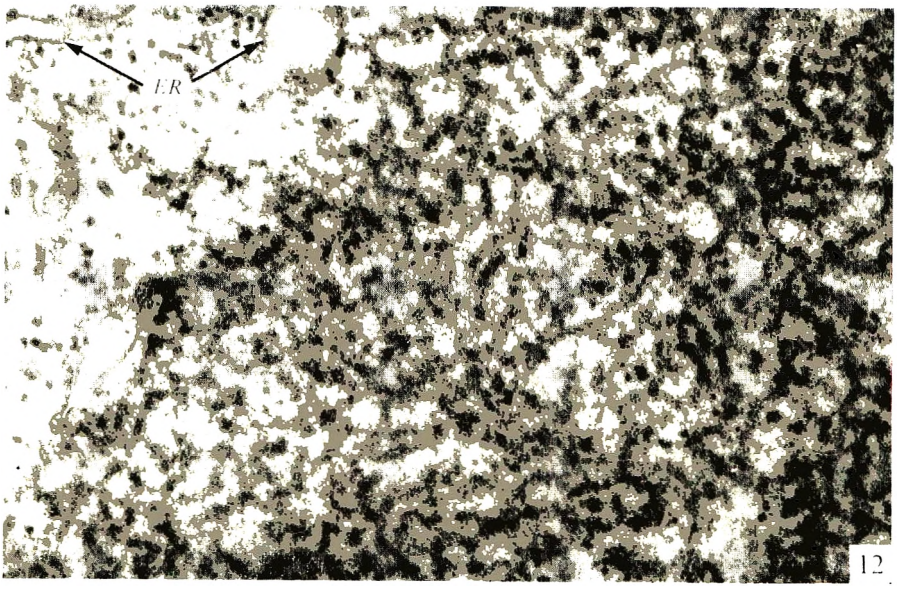
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PLATE 3

Fig. 5. Lymphocyte from child on second day of rash. Note inclusion body made up of islands of circular tubular structures. × 15,000.

Fig. 6. Lymphocyte from another child on second day of rash. Note two similar inclusions (arrows). × 19,000.

PLATE 4

Fig. 7. High-power view of inclusion shown in Fig. 5. Note circular profiles about 16 nm. diameter embedded in electron dense 'fuzzy' material. *N* = nucleus. × 82,000.

Fig. 8. Lymphocyte from a different child on second day of rash showing similar rather diffuse inclusion in proximity to endoplasmic reticulum though no functional association is suggested. *N* = nucleus. × 67,000.

PLATE 5

Fig. 9. Lymphocyte from patient with cancer of ovary. Note same type of cytoplasmic inclusions as shown in Figs. 1–4. × 37,000.

Fig. 10. Negative contrast preparation of disintegrated lymphocyte from another child on second day of rash. Cells had been frozen before preparation was made in P.T.A. Note structures resembling nucleocapsids of measles. × 154,000.

PLATE 6

Fig. 11. Electron micrograph through Ammon's horn of a baby mouse inoculated with Edmonston strain of measles virus. Note nucleoprotein tubules within the neuron and accumulations of ribosomes. *N* = nucleus. × 22,000.

Fig. 12. High magnification of Fig. 11. Note typical nucleoprotein tubules. *ER* = endoplasmic reticulum. × 52,000.

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Thermal sensations of secondary schoolchildren in summer

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SUMMARY

Summer thermal sensations of secondary schoolchildren in England are compared with those earlier published for winter. Heat stress, contrary to expectation, is seen to decrease during the warm months, and neutrality is found to increase by some 6° F. (3·5° C.) air temperature, which is more than double the increases observed for adults under similar circumstances.

INTRODUCTION

Seasonally varying thermal comfort requirements in England have been reported for adults (Hickish, 1955). Given that such differences also exist for other populations, such as schoolchildren, single yearly temperature recommendations may be misleading. Moreover, the prescription of minimum permissible thermal levels (Statutory Instruments, 1954) gives no indication of desirable conditions, particularly as relates to the warm months of the year.

Winter optima for schoolchildren were established earlier (Auliciems, 1969), showing thermal neutrality to occur in conditions very closely corresponding to those previously found for adults under similar rates of metabolic activity (Bedford, 1936), but being considerably in excess of legally required temperatures. The present report is designed to provide comparative information for the summer population of schoolchildren.

METHOD

Collection of data was identical with that previously reported for winter (Auliciems, 1969), but using children from 14 secondary schools in the Reading area. Atmospheric measurement included air temperature, radiation (globe thermometer), humidity (Assmann psychrometer) and rate of air movement (kata thermometer), while subjective thermal sensations were gained on a seven-point scale (see Table 1). Three separate measures of ambient warmth were employed for data analysis: dry-bulb temperature, corrected effective temperature (C.E.T.) and equivalent temperature (T_{eq}). The data were collected during late May and June in 1967.

RESULTS

The distribution of thermal sensations appears in Table 1, and the frequency of various degrees of warmth in Tables 2–4. As in the winter study, ventilation rates were very low despite frequently opened windows, with average air movement for

Table 1. *Distribution of thermal sensations*

Sensation	Numerical value	Frequency		Total
		Boys	Girls	
Much too warm	+3	16	16	32
Too warm	+2	59	35	94
Comfortably warm	+1	112	58	170
Comfortable	0	132	52	184
Comfortably cool	−1	95	29	124
Too cool	−2	39	9	48
Much too cool	−3	11	2	13
Total		464	201	665

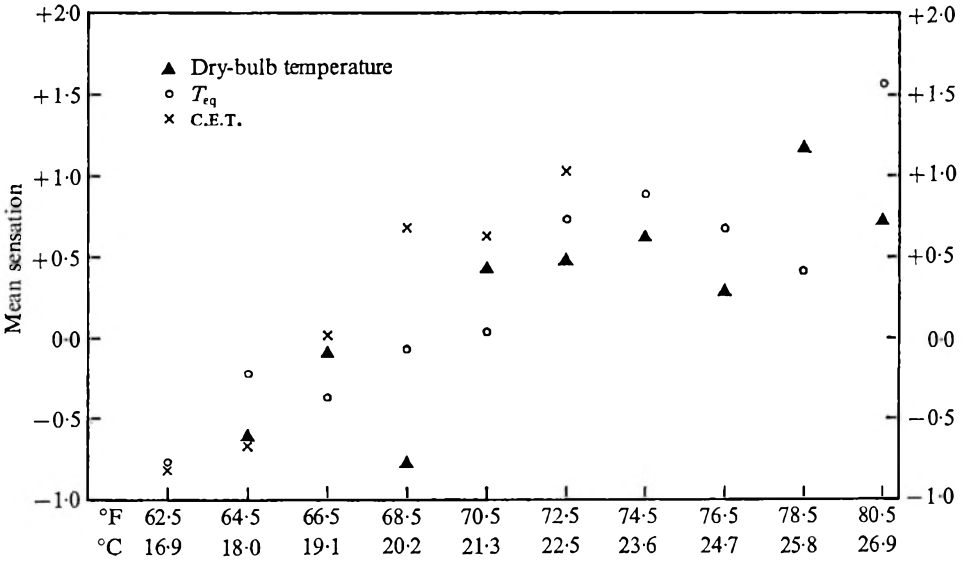

Fig. 1. Mean sensation against thermal levels.

the 36 sessions being 14 ft./min. (7 cm./sec.). Tables 2–4 also show the distribution of thermal sensations and means together with percentages of comfort for corresponding intervals of thermal levels.

Graphical representation of the sensation means against all three thermal measures is shown in Fig. 1. Regression analysis has been applied to individual scores and equations expressed in the form $Y = ax+b$, where Y is the thermal sensation, a and b constants and x thermal measure. The regression constants and the product moment correlation coefficients are shown in Table 5 and the optimum conditions (when $Y = 0$ or 'comfortable') in Table 6 together with those previously obtained for the children during winter and the earlier optima for light industrial workers.

Table 2. *Distribution of sensations in relation to dry-bulb temperatures*

Dry-bulb °F.	Dry-bulb °C.	Frequency of observations	Frequency of sensations +3	+2	+1	0	−1	−2	−3	Total	Mean sensation	Percentage Heat discomfort	Comfort		Cold discomfort
79·5–81·4	26·4–27·4	1	—	2	2	2	1	—	—	7	+0·71	29	71	59	0
77·5–79·4	25·3–26·3	2	8	12	13	6	7	—	—	46	+1·17	43	57		0
75·5–77·4	24·2–25·2	2	—	5	13	4	1	1	—	24	+0·83	21	75	69	4
73·5–75·4	23·1–24·1	1	2	4	3	5	3	—	1	18	+0·61	33	61		6
71·5–73·4	21·9–23·0	10	5	39	56	49	35	7	2	193	+0·49	23	72	75	5
69·5–71·4	20·8–21·8	12	14	25	68	65	40	11	1	224	+0·41	18	77		5
67·5–69·4	19·7–20·7	3	—	1	8	19	14	13	6	61	−0·79	2	67	69	31
65·5–67·4	18·6–19·6	2	2	4	4	15	10	3	2	40	−0·10	14	73		13
63·5–65·4	17·5–18·5	3	1	2	3	19	13	13	1	52	−0·62	6	67		27
Total		36	32	94	170	184	124	48	13	665		19	72		9

Table 3. *Distribution of sensations in relation to* T_{eq}

T_{eq} °F.	T_{eq} °C.	Frequency of observations	Frequency of sensations +3	+2	+1	0	−1	−2	−3	Total	Mean sensation	Percentage: Heat discomfort	Percentage: Comfort	Percentage: Cold discomfort
79·5–81·4	26·4–27·4	2	8	11	7	3	3	—	—	32	+1·56	59	41 } 58	0
77·5–79·4	25·3–26·3	2	—	3	8	5	5	—	—	21	+0·43	14	86 }	0
75·5–77·4	24·2–25·3	2	—	5	16	7	3	1	—	32	+0·66	16	81 } 70	3
73·5–75·4	23·1–24·2	7	10	27	39	26	15	—	1	118	+0·89	31	68 }	1
71·5–73·4	21·9–12·0	6	7	27	29	24	19	2	1	109	+0·72	31	66 } 76	3
69·5–71·4	20·8–21·8	10	4	15	57	69	48	18	3	214	+0·03	9	81 }	10
67·5–69·4	19·7–20·7	1	—	—	3	5	2	1	—	11	−0·09	0	91 } 69	9
65·5–67·4	18·6–19·6	3	2	4	8	26	16	13	7	76	−0·37	8	66 }	26
63·5–65·4	17·5–18·5	2	1	1	1	13	6	7	—	29	−0·24	7	69 } 67	24
61·5–63·4	16·4–17·4	1	—	1	2	6	7	6	1	23	−0·78	4	65 }	31
Total		36	32	94	170	184	124	48	13	665		19	72	9

Table 4. *Distribution of sensations in relation to* C.E.T.

C.E.T. °F.	C.E.T. °C.	Frequency of observations	Frequency of sensations +3	+2	+1	0	−1	−2	−3	Total	Mean sensation	Percentage: Heat discomfort	Percentage: Comfort	Percentage: Cold discomfort
71·5–73·4	21·9–23·0	5	8	19	28	12	9	1	—	77	+1·03	35	64	1
69·5–71·4	20·8–21·8	8	11	32	45	40	30	2	2	162	+0·63	27	71	2
67·5–69·4	19·7–20·7	7	6	28	41	32	18	3	1	129	+0·68	26	71	3
65·5–67·4	18·6–19·6	10	5	12	48	59	42	17	3	186	+0·01	9	80	11
63·5–65·4	17·5–18·5	5	2	2	6	35	18	19	6	88	−0·66	5	67	28
61·5–63·4	16·4–17·4	1		1	2	6	7	6	1	23	−0·78	4	66	30
Total		36	32	94	170	184	124	48	13	665		19	72	9

Table 5. *Regression constants and correlation coefficients*

Thermal measure	r	Constant a °F.	Constant a °C.	Constant b °F.	Constant b °C.
Dry-bulb	+0·32	0·125	0·225	−8·625	−4·614
T_{eq}	+0·40	0·133	0·240	−9·242	−4·976
G.E.T.	+0·39	0·194	0·350	−12·948	−6·731

Table 6. *Thermal optima*

	Schoolchildren					
	Summer		Winter (Auliciems, 1969)		Increase	
	°F.	°C.	°F.	°C.	°F.	°C.
Dry-bulb	69·0	20·6	62·7	17·1	6·3	3·5
T_{eq}	69·5	20·8	61·7	16·5	7·8	4·3
C.E.T.	66·7	19·3	60·8	16·0	5·9	3·3
	Adults					
	Summer (Hickish, 1955)		Winter (Bedford, 1936)		Increase	
	°F.	°C.	°F.	°C.	°F.	°C.
Dry-bulb	66·8	19·3	64·7	18·2	2·1	1·1
T_{eq}	65·9	18·8	62·3	16·8	3·6	2·0
G.E.T.	64·4	18·0	61·7	16·5	2·7	1·5

DISCUSSION

Perhaps the most significant result to emerge from the present analysis is that less heat discomfort is evident during the warm months than the cool ones. During the latter, fully 31 % of the subjects had reported being either 'much too warm' or 'too warm' (Auliciems, 1969), while in the summer heat discomfort was reduced to only 19 % (see Tables 2–4). The present data do not permit the establishment of a satisfactory comfort zone, but it would appear that the lower limits for approximately 70 % of the sample can be located above 65·5° F. (18·6° C.) with all three thermal measures, and the upper limits at 77·4° F. (25·2° C.) dry-bulb and T_{eq}, and 71·4° F. (21·8° C.) C.E.T. The previously employed comfort criterion of 60 % in winter was exceeded at almost all thermal levels and it must be concluded that the overall conditions in the schools were thermally preferable during summer.

Of particular interest also is the considerably greater increase of optima in comparison with those of adults (see Table 6). With all thermal measures the differences are at least double in magnitude, but the reasons are far from obvious. It may be suggested that children tend to wear more clothing during cold weather than adults, which removed in summer would lead to relatively greater increases in thermal requirements. If so, however, then being more heavily dressed, the children should have also preferred considerably lower temperatures in winter,

particularly in view of their higher basal metabolic rates. Thus unless the metabolic heat production of adults had been higher owing to comparatively increased rates of activity at the time of testing, which does not appear to have been the case, the differential winter–summer increases must have resulted from some more subtle and less easily identifiable process of behavioural or physiological adaptation to seasonal changes.

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Salmonella dublin abortion in cattle

I. Observations on the serum agglutination test

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SUMMARY

The somatic and flagellar serum agglutinin titre were determined in paired samples obtained from seventy-seven cases of bovine abortion associated with *Salmonella dublin* infection. The cases could be divided into four serological groups with an active infection being demonstrated in most cases. The serum agglutination test was shown to be a relatively specific diagnostic test but was of more limited value in the retrospective identification of convalescent cases.

INTRODUCTION

S. dublin is now a well known cause of bovine abortion. The abortion may be associated with symptoms of dysentery or it may be the only clinical sign. The literature on the subject has been reviewed (Hinton, 1971*b*). During the last decade the incidence of these abortions has increased. Salmonellas, mainly *S. dublin*, were isolated from 9 (0·19 %) of 4785 bovine abortions investigated by the Veterinary Investigation Service in England and Wales between November 1959 and October 1961 (Hughes, 1964) and from 502 (2·7 %) of the 18,382 abortions examined during 1970 (Report, 1971).

Diagnosis is usually based on the isolation of the salmonella from foetal material while a preliminary report has indicated that the serum agglutination test is a useful adjunct to diagnosis especially when both the somatic (O) and the flagellar (H) antibody titres are determined (Hinton, 1971*a*).

This paper records detailed results obtained when using the serum agglutination test.

MATERIAL AND METHODS

Clinical cases

These were encountered during the course of routine investigations of bovine abortion material. The main results are drawn from a total of 111 abortions associated with *S. dublin* infection. In addition, convalescent samples only were collected from a further 10 cases. None of the cows had been inoculated with the live rough *S. dublin* vaccine (Mellavax, Burroughs Wellcome & Co.). Serum O and H agglutination titres were determined in paired samples from seventy-seven

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cases of abortion. Abortion was the only clinical sign in 64 of the cases. Seven were associated with dysentery and six cows were off-colour or had diarrhoea at the time of abortion. The first of the pair of serum samples was obtained within five days of the abortion while at least 7 days elapsed between the samplings though all the second samples were collected within 26 days of abortion.

Convalescent serum samples were obtained, 30–330 days after abortion, from 40 cases. These included 25 out of the 77 cases from which paired samples were examined.

Serum agglutination test

Serum was examined by the tube agglutination test. Doubling dilutions of the serum were made in 0·5 % phenol-saline and these were incubated in a water bath at 48° C with a standard suspension of *S. pullorum* O (1, 9, 12) and *S. dublin* H (gp) antigen obtained from the Central Veterinary Laboratory, Weybridge. The H titre was read after four hours and the O titre after overnight incubation. The endpoint was taken as the highest dilution giving 50 % agglutination and the titre expressed as a reciprocal of that dilution. A significant change in titre has been taken to be at least a four-fold change, either up or down.

In order to test the specificity of the agglutination test paired serum samples from 690 abortions from which *S. dublin* was not isolated were screened for *S. dublin* O and H agglutinins. For convenience only the H titres are recorded in the results. The abortions occurred in either Breconshire, Carmarthenshire, S. Cardiganshire or Pembrokeshire between July 1971 and February 1972. *Brucella abortus* was not isolated from any of the cases; the series included 62 cases of mycotic infection and 15 cases associated with *Corynebacterium pyogenes*.

INTERPRETATION OF THE SERUM AGGLUTINATION TEST

In salmonella serology there is no standardization of either techniques or antigen production. This fact makes it difficult, not only to compare the results of other workers, but also to define acceptable diagnostic criteria for the serological diagnosis of infection.

One of the problems of using a salmonella agglutination test in adult cattle is the presence of agglutinating antibody in the sera of apparently normal cattle. The reason for this has not been fully explained though recently Sharpe & Reiter (1971) demonstrated common antigens between the salmonellas and the butyrio-vibrios which are found in the normal ruminal micro-flora. Similarly Protell *et al.* (1971) recorded that, owing to a dysglobinaemia, 11 of 40 human patients with chronic active liver diseases had raised serum salmonella agglutinins. This interesting finding has yet to be evaluated in the context of bovine liver disease (e.g. fascioliasis) but it may prove to be of some significance.

In his review, Gibson (1965) drew on the results of a number of workers and suggested that the sera of normal cattle seldom show an O titre above 40 or an H titre above 160. A suggested guide for interpretation of the serum agglutination test, based on Gibson (1965) and observations in this study is given in Table 1 and this is used throughout this paper.

Table 1. *The interpretation of the* Salmonella dublin *serum agglutination test*

	Test result		
	Negative	Doubtful	Positive
Somatic (O) titres	⩽ 20	40	⩾ 80
Flagellar (H) titres	⩽ 40	80–160	⩾ 320

RESULTS

The examination of paired samples from seventy-seven cases

The full results are listed in Table 2 and summarized in Table 3. These show that the cases can be divided into four groups. In Group I there is a rise in either the O and/or H titre while in Group II a fall in H titre was recorded. In the third group there are diagnostic titres at both samplings but these show no significant change. In group IV diagnostic agglutinin titres do not develop.

About 80 % of cases showed significant changes in O or H titre and were classed in Groups I and II. In the 50 cases in Group I the O and H titre rose significantly in 33, the O only in seven and the H only in 10. The distribution of the O and H titres in both the first and second samples are listed in Table 4 and the changes in O and H titre are summarized in Table 5.

The median change in H and O titre was 16- and 8-fold and was four-fold or more in 43 (86 %) and 40 (80 %) of cases respectively. In the seven cows (nos. 6, 33, 79, 82, 102, 106, 107) in which the H titres did not change significantly the O titres rose between four and 32-fold.

In 10 Group I cases (nos. 19, 21, 31, 37, 51, 56, 65, 69, 97, 100) the changes in O titre were between a two-fold rise and a four-fold fall. The final O titres ranged between 20 and 640. In eight of the 10 it was 80 or more in one or both samples. In the other two both titres were ⩽ 20. The final H titre in no. 37 was 1280 while in no. 100 it was only 160 some 26 days after abortion.

Eleven cases (nos. 18, 23, 24, 30, 36, 40, 47, 60, 63, 71, 76) were classified in Group II. The H titres fell by between four and 64-fold, the median change being eight-fold. The behaviour of the O titres was variable and showed between a four-fold and an eight fold rise in titre. The initial H titres, which exceeded 5120 in nine cases, ranged between 640 and 20,480, while the H titres of the second samples were between 160 and 2560 with a median of 640. In two cases (nos. 36, 47) the final H titres were 160. The corresponding O titres were 80, indicating that if this was the only sample examined, the cases would have been classified as doubtful positives (see Table 1) even though there had been an active infection.

In seven cases in Group III (nos. 2, 39, 42, 44, 46, 61, 92) the H titres were all between 320 and 5120 and the O titres were all 160 or more except in case no. 39 when they were 40 and 80 respectively. In the eighth case (no. 109) the H titres were 160 and 320 and the O titres only 20. These titres indicate that this case falls into an intermediate position between Groups III and IV.

Group IV comprised eight cases (nos. 8, 48, 49, 64, 80, 84, 93, 104) which did not

Table 2. *The serological findings in seventy-seven cases of abortion associated with* Salmonella dublin *infection*

Case no.	Clinical findings*	Day of sampling†		Serum O titre		Serum H titre		Change in titre‡		Sero-logical Group
		1st	2nd	1st	2nd	1st	2nd	O	H	
2	A	1	17	320	320	320	320	0	0	III
3	A	1	16	40	160	80	2,560	+4	+32	I
6	A	4	23	80	320	2,560	2,560	+4	0	I
7	A	4	23	40	320	640	10,240	+8	+16	I
8	A	3	21	20	< 40	20	< 40	0	0	IV
11	A	2	9	< 10	160	20	2,560	+16	+128	I
14	A	1	18	< 20	320	< 20	5,120	+32	+512	I
15	A	1	30	40	160	80	1,280	+4	+16	I
18	D+A	4	24	320	160	10,240	1,280	−2	−8	II
19	A+M	1	20	80	160	40	1,280	+2	+32	I
21	A+S	3	19	80	80	160	640	0	+4	I
22	A	−2	11	80	320	160	5,120	+4	+32	I
23	A	5	19	160	40	20,480	320	−4	−64	II
24	A+M	1	17	320	320	10,240	640	0	−16	II
29	A	1	15	40	160	320	10,240	+4	+32	I
30	A	2	26	80	640	10,240	2,560	+8	−4	II
31	A	1	10	80	160	< 20	640	+2	+64	I
33	A	1	11	80	640	1,280	2,560	+8	+2	I
34	A	1	8	40	160	20	5,120	+4	+256	I
36	D + PLC	1	8	160	80	640	160	−2	−4	II
37	A	1	14	< 20	20	< 20	1,280	+2	+128	I
39	A	4	21	40	80	320	640	+2	+2	III
40	A	5	19	320	80	5,120	1,280	−4	−4	II
41	A	1	19	20	80	160	1,280	+4	+8	I
42	A	1	18	160	320	320	320	+2	0	III
44	A	1	15	320	640	5,120	5,120	+2	0	III
45	A	1	20	80	640	20	2,560	+8	+256	I
46	A	1	24	160	160	640	1,280	0	+2	III
47	A	2	25	40	80	1,280	160	+2	−8	II
48	A	1	13	< 20	< 20	< 20	< 20	0	0	IV
49	A	1	22	< 20	< 20	< 20	< 20	0	0	IV
50	A+S	3	13	40	640	160	1,280	+16	+8	I

Table 2 (*cont.*)

Case no.	Clinical findings*	Day of sampling†		Serum O titre		Serum H titre		Change in titre‡		Serological Group
		1st	2nd	1st	2nd	1st	2nd	O	H	
51	A	1	14	80	160	20	1,280	+2	+64	I
53	A	1	12	40	160	< 20	640	+4	+64	I
54	A	1	20	40	160	80	640	+4	+8	I
55	D+A	1	14	< 20	640	320	1,280	+64	+4	I
56	D+A	1	18	40	80	160	1,280	+2	+8	I
57	A+D	1	11	40	640	20	1,280	+16	+64	I
58	A	1	12	80	640	40	10,240	+8	+256	I
60	A	3	16	320	320	5,120	640	0	−8	II
61	A	5	14	320	320	1,280	1,280	0	0	III
63	A	2	17	640	320	5,120	1,280	−2	−4	II
64	A	1	14	< 20	< 20	< 20	< 20	0	0	IV
65	A	1	25	640	640	640	2,560	0	+4	I
66	A	1	16	40	640	40	1,280	+16	+32	I
69	A	1	14	320	640	80	1,280	+2	+16	I
70	A	3	17	10	80	20	640	+8	+32	I
71	A	1	14	80	40	10,240	2,560	−2	−4	II
73	A	1	15	< 20	160	< 20	2,560	+16	+256	I
75	A	1	15	20	80	80	640	+4	+8	I
76	A	1	14	80	160	5,120	640	+2	−8	II
79	A	3	14	40	320	320	320	+8	0	I
80	A	1	11	< 20	< 20	< 20	< 20	0	0	IV
81	A	1	20	< 20	160	40	640	+16	+16	I
82	A	1	19	40	160	2,560	1,280	+4	−2	I
83	A	1	15	40	320	160	2,560	+8	+16	I
84	A	1	17	< 20	< 20	< 20	< 20	0	0	IV
85	A	1	17	< 20	160	80	640	+16	+8	I
86	A+D	1	15	< 20	80	20	320	+8	+16	I
87	A	1	26	< 20	160	80	2,560	+16	+32	I
88	A	1	18	160	640	320	5,120	+4	+16	I
90	A	1	18	< 20	160	80	640	+16	+8	I
91	A	1	18	80	640	80	1,280	+8	+16	I
92	A	1	17	320	640	640	1,280	+2	+2	III

Table 2 (*cont.*)

Case no.	Clinical finding*	Day of sampling†		Serum O titre		Serum H titre		Change in titre‡		Serological Group
		1st	2nd	1st	2nd	1st	2nd	O	H	
93	A	1	17	< 20	< 20	< 20	< 20	0	0	IV
94	D+A	2	9	< 20	160	< 20	1,280	+16	+64	I
95	A	2	15	< 20	80	80	320	+8	+4	I
96	A+M	1	13	< 20	80	160	2,560	+8	+16	I
97	A	2	17	160	40	640	5,120	−4	+8	I
98	A+M	1	23	40	320	640	2,560	+8	+4	I
99	A	2	11	< 20	640	< 20	5,120	+64	+512	I
100	A	2	26	< 20	20	40	160	+2	+4	I
102	A	1	16	20	80	2,560	1,280	+4	−2	I
104	A	1	10	20	20	40	40	0	0	IV
106	A	3	15	20	320	320	320	+16	0	I
107	A	1	16	20	640	640	1,280	+32	+2	I
109	A	1	15	20	20	160	320	0	+2	III

* A = abortion, D = dysentery, S = scour (diarrhoea), M = malaise, PLC = premature live calf.
† Day 1 is the first 24 hr. after abortion.
‡ Degree of change 2-fold, 4-fold, etc., 0 = no significant change.

Table 3. *A summary of the serological response in seventy-seven cases of abortion associated with* Salmonella dublin *infection*

	Serological response	No. cases	Proportion (%)
Group I	Cases with a significant rise in O and/or H titre	50	65
Group II	Cases with a significant fall in H titre	11	14·2
Group III	Cases with diagnostic titres but which showed no significant change	8	10·4
Group IV	Cases with titres of ⩽ 80 at both samplings	8	10·4

Table 4. *A summary of the O and H titres in the first and second samples from fifty cases of* Salmonella dublin *abortion classified as Group I*

	No. of cases with titres					Proportion (%) of samples*			Titres	
	⩽ 20	40	80	160	⩾320	—	d	+	Range	Median
Initial H titre	14	5	10	7	14	38	34	28	⩽ 20–2,560	80
Final H titre	—	—	—	1	49	—	2	98	160–10,240	1,280
Initial O titre	21	15	10	2	2	42	30	28	⩽ 20–640	40
Final titre	2	1	9	17	21	4	2	94	⩽ 20–640	160

* See Table 1 for interpretation.

Table 5. *A summary of the changes in O and H titres in fifty cases of* Salmonella dublin *abortion classified as Group I*

	Changes in titre between samples											
	−4	−2	0	+2	+4	+8	+16	+32	+64	+128	+256	+512
H titres		2	3	2	6	8	9*	7	5	2	4	2
O titres	1	0	2	7	13	12*	11	2	2			

* = Indicates the median change in titre.

show a serological response as judged by the S.A.T. The placenta was positive on direct culture in two and selenite enrichment was necessary for the isolation of *S. dublin* in the remainder.

Serological findings in convalescent cases

Forty cases were examined between 30 and 330 days after an abortion associated with *S. dublin* infection. The findings are summarized in Table 6.

These results show that as time passes the serum titres generally fall so that by 150 days or more after abortion only 30% have an O titre of ⩾ 80 and 40% an H titre of ⩾ 320. Faeces samples were cultured from 30 of the cases examined 45 days or more after abortion. Seven yielded *S. dublin* and in five of these the H titre was 320 or more.

Table 6. *The serum titres in convalescent abortion cases from which* Salmonella dublin *was isolated*

Days after abortion	No. of cases	Including cases listed in Table 2	Proportion (%) with titres in the ranges: Somatic O < 20	Somatic O 20–40	Somatic O ⩾ 80	Flagellar H ⩽ 40	Flagellar H 80–160	Flagellar H ⩾ 320
30–40	7	15, 45, 46, 54, 70, 107	—	—	100	—	—	100
45–120	19	2, 18, 24, 36, 47, 53, 73, 81, 88, 90, 91, 95, 102, 106	10·5	31·5	58	10·5	47·5	42
145–330	14	3, 11, 71	21·5	50	28·5	28·5	28·5	43

Table 7. *The screening of paired serum samples from* 690 *cases of abortion which did not yield* Salmonella dublin *on culture*

Material cultured at the first sampling	H titre recorded in the paired sera ⩽ 40*	80–160	160–320	⩾ 320	Total
Foetus and/or placenta	489	10	2(*a*)	1(*a*)	502
Vaginal mucus	122	—	—	2(*b*)	124
No cultures	62	2	—	—	64
Total	673	12	2	3	690

(*a*) and (*b*) see explanation in text, p. 467.

* These include 29 cases in which a titre of 80 was recorded in one sample only.

Screening of paired sera from 690 *cases for* Salmonella dublin *agglutinins*

Paired serum samples from 690 cases of abortion which did not yield *S. dublin* or *Br. abortus* on culture were screened for *S. dublin* agglutinins. The results are listed in Table 7 and these indicate that 97·5% of cases were negative while 2% had doubtful and 0·5% positive titres. In the three cases marked under (*a*) in the Table the titres did not change so that it is possible that these are residual titres. In two of the three cases selenite enrichment was employed. In the two cases marked under (*b*) one showed a Group I and the other a Group II response. In both cases a vaginal swab only was cultured on solid medium and no selenite enrichment was used. In all probability, therefore, there was a failure in the bacteriological technique rather than a lack of specificity of the agglutination test.

DISCUSSION

Loizelier (1945), Sellers & Sinclair (1953), Coutard & De Saint-Aubert (1969) and Harbourne, Randall, Luery & Wallace (1972) all recorded that significant agglutinin titres may be found in the serum of cows after a salmonella abortion. Le Guilloux (1969) and Cottereau, Rancien & Sendral (1970) examined paired serum samples and noted rising titres in four of five and six of nine cases respectively while a preliminary report by the author (Hinton, 1971*a*) proposed that *S. dublin* abortions could be divided into four groups on the basis of the serological findings.

The cases in Groups I and II are relatively easily explained as they obviously confirm an active infection. In Group I the change in H titre usually exceeded that of the O. A significant change in O and H titre was noted in 40 and 43 of the 50 cases respectively. The H titre of the second sample was $\geqslant$ 320 in 49 of 50 cases. The O titre was $\geqslant$ 80 in 47 cases though in three it was $\leqslant$ 40 indicating that on occasions it was of no diagnostic value. In Group II the H titres fell and this is due to the fact that in salmonella infections the flagellar titres often reach high levels fairly soon after infection and then fall again relatively rapidly. This has also been seen in experimental *Br. abortus* abortion (Thomsen 1950). In some Group II cases (e.g. nos. 30, 47, 76) the O titres continued to rise indicating that the O and H agglutinins develop at different rates.

The findings in Groups I and II suggest that the peak in the agglutinin titres develops towards the end of the first week. On the other hand there is evidence to suggest that in cases of dysentery associated with *S. dublin* infection the peak titres are not reached until 10–14 days after the onset of clinical signs (Field, 1948; Gibson, 1958).

In the cases in Group III there are diagnostic titres at both samplings but these do not show any apparent change. In these cases the abortion may have occurred in a carrier cow which had high residual titres and in which *S. dublin* was isolated from the products of conception. On the other hand there may have been an active infection with diagnostic titres developing before abortion, but unlike Groups I and II, they did not change during the sampling period. Alternatively

the titres may have continued to rise for a short while after the abortion but fell again so that by the second sampling they were at the same level as they were originally.

In Group IV the explanations are more speculative. The first is that there may have been an active infection which was not demonstrated by the S.A.T., while secondly the abortion may have occurred in a carrier cow which had low residual titres but in which there was congenital infection of the placenta or foetus. The third explanation is that the abortion may have precipitated a transient activation of the latent carrier state and lastly, extraneous contamination of the sample may have occurred and the isolation has no clinical significance whatsoever.

The explanations propounded for the cases in Groups III and IV indicate that, as with many infectious diseases, diagnosis is not always straightforward and that several factors have to be considered before an opinion can be given.

The screening of paired serum samples from nearly 700 abortions from which *S. dublin* was not isolated indicated that the agglutination test was unlikely to give many false positive results. The interpretation of the titres was discussed in an earlier section though no mention was made of the relative merits of either the O or the H titres for diagnosis. In his study of *S. dublin* abortion Le Guilloux (1969), who used different serological techniques, concluded that the O agglutinins were of more diagnostic value than the H and this view is in agreement with that of Huckstep (1962) in his consideration of the serology of typhoid fever in man. On the other hand the results presented in this paper suggest that though the H titres were marginally superior to the O titre for diagnostic purposes it is important that both O and H titres are always determined.

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Salmonella dublin abortion in cattle

II. Observations on the whey agglutination test and the milk ring test

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SUMMARY

The serum and whey agglutination test were compared on paired samples from thirty-five cases of bovine abortion associated with *Salmonella dublin* infection. The whey test proved nearly as useful as the serum test for confirming an active infection though it was only practicable to examine the whey for flagellar antibodies. *S. dublin* was isolated from nearly half of the milk samples obtained within the first week of abortion but none of those collected after the fourth week. The whey test proved of no value in retrospective identification of abortion cases. The trial using the milk ring test was disappointing.

INTRODUCTION

During the course of clinical investigations of *S. dublin* abortion cases it was noted that high agglutinin titres to the flagellar antigens could often be detected in the whey. The effect of the method of preparation of whey on the *S. dublin* agglutinin titre has been reported elsewhere (Hinton, 1972). This paper records the changes that occur in the whey titres following a salmonella abortion together with details of a milk ring test (M.R.T.) using a stained *S. dublin* antigen.

MATERIALS AND METHODS

Clinical cases

These were encountered during the course of routine investigations of bovine abortion material, as described in the previous paper (Hinton, 1973). Abortion was the only clinical sign in 31 of the cows. In cases 18, 21 and 56 (see Table 1) there were also symptoms of dysentery while case no. 24 was ill after abortion though she did not develop diarrhoea.

When milk was cultured 5 ml. was incubated with 5 ml. double strength selenite F (Oxoid Ltd., London, S.E. 1) at 37° C. One subculture was made after 18–24 hr. onto either deoxycholate citrate agar (Hynes modification) (Oxoid) or MacConkey medium (Oxoid) containing 1/25,000 brilliant green.

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Table 1. *Changes in the serum and whey flagellar agglutination titres in 35 cases of* Salmonella dublin *abortion*

Case no.	Day collected*		Serum H titre		Whey H titre		Change in titre†	
	1st	2nd	1st	2nd	1st	2nd	Serum	Whey
18	4	24	10,240	1,280	640	160	−8	−4
19	1	20	40	1,280	< 20	160	+32	+16
21	3	19	160	640	20	80	+4	+4
22	−2	11	160	5,120	20	640	+32	+32
23	5	19	20,480	320	1,280	160	−64	−8
24	1	17	10,240	640	1,280	160	−16	−8
31	1	10	< 20	640	< 20	160	+64	+16
34	1	8	20	5,120	< 20	640	+256	+64
36	1	8	640	160	640	20	−4	−32
39	4	21	320	640	80	80	+2	0
40	5	19	5,120	1,280	640	80	−4	−8
41	5	19	5,120	1,280	640	1,280	−4	+2
42	3	18	1,280	320	320	40	−4	−8
44	3	15	10,240	5,120	5,120	160	−2	−32
47	4	25	1,280	160	2,560	40	−8	−64
54	3	20	1,280	640	320	< 20	−2	−32
56	1	18	160	1,280	20	160	+8	+8
60	3	16	5,120	640	320	80	+8	−4
63	4	10	5,120	640	160	< 20	−8	−16
71	1	14	10,240	2,560	320	160	−4	−2
73	3	15	< 20	2,560	< 20	640	+256	+64
79	3	14	320	320	80	160	0	+2
80	5	11	< 20	< 20	< 20	< 20	0	0
81	1	20	40	640	< 20	80	+16	+8
82‡	1	19	2,560	1,280	160	1,280	−2	+8
88	1	18	320	5,120	20	5,120	+16	+256
90	1	18	80	640	< 20	80	+8	+8
91	1	18	80	1,280	80	640	+16	+8
92	1	17	640	1,280	1,280	1,280	+2	0
98	1	23	640	2,560	40	160	+4	+4
100	2	26	40	160	< 20	< 20	+4	0
102	1	16	2,560	1,280	160	80	−2	−2
106	3	15	320	320	160	160	0	0
107	3	16	640	1,280	80	80	+2	0
109	1	15	160	320	< 20	< 20	+2	0

* Day 1 is the first 24 hr. after abortion.
† Degree of change two-fold, four-fold, etc. 0 = No significant change.
‡ In the same period the serum O titre rose from 40 to 160.

Whey preparation

The milk was drawn, usually without aseptic precautions, from one or two quarters of the infected cow's udder. The whey was prepared by adding two drops of cheese-making rennet to 2 ml. of milk. This was incubated in a water bath at 52° C for 20–30 min. and then centrifuged at 2000 rev./min. for 10 min. to separate the curd from the whey.

Table 2. *Summary of changes in the serum and whey flagellar titres in thirty-five cases of* Salmonella dublin *abortion*

Group	Changes in H titre*		Case no.	Total
	Serum	Whey		
A	Rise	Rise	19, 21, 22, 31, 34, 56, 73, 81, 88, 90, 91, 98	12
B	Fall	Fall	18, 23, 24, 36, 40, 42, 47, 60, 63	9
C	Rise	NSC	100	1
D	NSC	Rise	82	1
E	Fall	NSC	41, 71	2
F	NSC	Fall	44, 54	2
G	NSC	NSC	39, 79, 80, 92, 102, 106, 107, 109	8

NSC = No significant change in titre, i.e. twofold or less.
* Significant rise or fall in titre is equivalent to fourfold or more.

Serum and whey agglutination test

Serum was examined by the tube agglutination test, by the technique described in the previous paper (Hinton, 1973). The whey was examined in a similar manner, but for the presence of flagellar antibodies only.

Milk ring test

A smooth, motile strain of *S. dublin*, isolated from a case of abortion, was seeded into nutrient broth, already at 37° C, and incubated at that temperature for 36 hr. The culture was killed by the addition of 0·5 % formalin. The bacterial cells were harvested by centrifugation, stained with a 1 in 5 solution of Harris' haematoxylin and then resuspended in 0·5 % phenol-saline to give a solution with a 4 % packed cell volume. The test was carried out as recommended by Alton & Jones (1967) on 59 samples in which the whey titre was determined concurrently. About half the samples were from apparently normal cows while the rest were from abortion cases.

RESULTS

It was frequently found that the somatic titres in the whey were very low and that it was difficult to assess the degree of agglutination, consequently no whey O titres are given in these results. However, when they were carried out the titres were usually less than 80 though titres up to 320 were recorded.

A comparison of serum and whey agglutination titres in abortion cases

The full results obtained are given in Table 1 and summarized in Table 2. The cases can be divided into seven groups depending on the changes of the serum and whey titres. In Groups A to F (Table 2) the fact that either a rise or a fall in titre was recorded was related to the time of collection (Table 1) of the first sample relative to the abortion. In 10 of the 14 cases showing a significant rise in serum or whey titre, the first sample was collected within 24 hr. of the abortion while in 10 of the 13 cases, in which there was a significant fall in titre, the first samples were collected within three to five days of the abortion.

Table 3. *Relation between the changes in the serum and whey* Salmonella dublin *whey flagellar titres in four cases of abortion in which serial samples were examined*

Case no.	Day sample collected*	Serum titre O	Serum titre H	Whey titre H
47	2	40	1,280	—
	4	40	1,280	2,560
	9	80	320	320
	19	80	320	20
	25	80	160	40
	45	40	160	20
73	1	< 20	< 20	—
	3	< 20	< 20	< 20
	8	20	5,120	1,280
	15	160	2,560	640
	45	160	320	20
81	1	< 20	40	< 20
	4	80	320	320
	6	160	1,280	320
	8	—	—	640
	20	160	640	80
	90	20	80	< 20
98	1	40	640	40
	3	40	5,120	1,280
	7	160	10,240	320
	23	320	2,560	160
	60	160	640	< 20

* Day 1 is the 24 hr. after abortion.

In the 12 cases in Group A the median rise in whey and serum titre was 16-fold with the changes varying between four and 256-fold. The rise in serum and whey titre was of the same order in five of the cases while in only one case (no. 88) did the degree of change in the whey titre exceed that of the serum, and this was because the whey titre was very low (20) at the time of abortion. In this group the final serum titres ranged between 640 and 5120 with the median at 1280, while in the whey, the range was between 80 and 5120 with the median at 160. In no case did the final whey titre exceed that of the serum though in case no. 88 the titres were the same at 5120.

In Group B both the serum and whey titre showed a significant fall. This ranged between four and 64-fold. The change in titre was greater in the whey in all except four cases (nos. 18, 23, 24, 60). In these the initial serum titres were high, being in the range 5120 to 20,480.

In Groups C, D, E and F only one or other of the serum or whey titres changed significantly. The titres not showing a significant change were in the range 160 to 10,240, excepting in case no. 100. In this no whey agglutinins were detected. The value of examining serum samples for O antibodies was confirmed by case no. 82; the serum H titre showed no significant change while the O titre rose from 40 to 160 during the same period.

Table 4. *The serum and whey flagellar agglutination titres in convalescent* Salmonella dublin *abortion cases*

Serum flagellar titre	Whey flagellar titre ⩽ 20	40	80	Total
⩽ 40	3			3
80–160	8			8
⩾ 320	3	2	1	6
Total	14	2	1	17

Table 5. *A comparison between the* Salmonella dublin *whey flagellar titre and the milk ring test score using a stained* Salmonella dublin *antigen*

MRT score	Whey H agglutination titre < 20	20–40	80–160	320–640	⩾ 1280	Total
−	8	3		1	2	14
+	9		1			10
+ +	6	3	3	2	4	18
+ + +	10	3	3	1		17
Total	33	9	7	4	6	59

In Group G the examination of paired serum and whey titres did not actually confirm an active salmonella infection though in six of the eight cases the maximum serum and whey titres ranged between 320 and 2560, and 80 and 1280 respectively. No serum and whey agglutinins were recorded in case No. 80 despite the fact that *S. dublin* was recovered from the foetus. A number of cases similar to this were identified in the full series of 111 cases and they probably represent transient excretion in the latent carrier animal, possibly activated by the stress of abortion. This feature of *S. dublin* infections will be discussed elsewhere.

Four or more samples of serum and whey were compared in four cases. The results are given in Table 3 and show that the changes in the whey titres follow those of the serum fairly closely though at a lower level.

The whey titre in convalescent cases of Salmonella dublin *abortion*

Seventeen cows were examined between 40 and 180 days after the abortion. The H titres of the serum and whey are given in Table 4. In 14 of the 17 cases the whey titres were 20 or less while in 11 the serum titre was 160 or less. *S. dublin* was cultured from faeces of four of the cows and in each case the whey titre was 20 or less.

The milk ring test

The results are summarized in Table 5 and show that the correlation between the M.R.T. score and the whey titre was not very good since 25 of the 45 samples with a positive M.R.T. had whey titres of less than 20 while three whey samples, which had titres between 640 and 2560, were negative to the M.R.T.

Table 6. *The isolation of* Salmonella dublin *from milk, vaginal mucus and faeces from* 49 *cases of abortion*

S. dublin isolations			Days after abortion				Total
Milk	VMS	Faeces	0–5	6–10	1–25	26†	
+	+	+	11	1	0	0	12
+	+	−	0	1	3	0	4
+	−	+	0	0	0	0	0
+	−	−	0	0	1	0	1
−	+	+	5	4	4	0	13
−	−	+	3	1	4	2	10
−	+	−	1	4	4	0	9
−	−	−	0	3	13	13	29
Total examined			20	14	29	15	78
Proportion of samples positive (%)							
Milk				55	14·3	13·7	—
VMS				85	71·5	38	—
Faeces				95	43	27·3	13·3

Table 7. *The relationship between the presence of* Salmonella dublin *in milk and the whey agglutination titre*

S. dublin in milk	No. examined	Whey agglutination titres			
		⩽ 20	40	80–160	⩾ 320
+	14	0	1	4	9
−	58	25*	2	16	15

* Includes 10 samples obtained 40 or more days after abortion and six samples from cows which did not develop serum or whey agglutination titres.

The isolation of Salmonella dublin *from milk*

Samples of milk, vaginal mucus and faeces were collected on 78 occasions from 49 cases. The results of culture are listed in Table 6. Over half the milk samples yielded *S. dublin* during the first 5 days after abortion, but the proportion of positive samples fell to nil by the end of the fourth week. A similar trend was noted in the case of vaginal mucus and faeces except that in 13·3% of the cases faecal excretion persisted beyond 4 weeks.

The whey agglutination titre was determined in 72 of the 78 milk samples. The distribution of the titres in both culturally positive and negative milks is listed in Table 7. The findings suggest that when *S. dublin* is isolated a whey titre of 80 or more is to be expected. A titre of 80 or more was also found in about half of the samples which were negative for *S. dublin* and this indicates that the presence of the organism is not essential for the appearance of the agglutinins in the milk.

DISCUSSION

The whey agglutination test

The results given in this paper indicate that when paired samples are examined the whey test is often as useful as the serum agglutination test for confirming that an abortion has been associated with an active *S. dublin* infection.

An analysis of the changes in the titres indicate that, when both are increasing, the rate of change of the serum titre often exceeds that of the whey, so that the serum to whey ratio either increases or at least remains the same. Conversely when the titres are falling, the rate of change in the whey was usually greater than that of the serum, except in cases in which the initial serum titre was very high.

A suggested interpretation for the serum agglutination test was given in the previous paper (Hinton, 1973). As far as the agglutinins in whey are concerned there appears to be no information about diagnostic titres. In brucella infections in non-vaccinated cattle, a positive result in the whey is taken at a titre of + +/10 while the corresponding titre in serum is + + + +/40 (Morgan, 1967). This represents an approximately four-fold difference between titres, and if a difference of this order is applied to the salmonella agglutination titres, then a whey titre of 80 or more will be diagnostic, with a doubtful range between 20 and 40.

Apart from the three cases (nos. 80, 100 and 109) in which there was no evidence of agglutinins in either sample, a whey titre of 80 or more was recorded in at least one of each pair of whey samples. This finding coupled with the fact that whey titres of less than 80 were found in only nine (20%) of the 43 samples collected after the third day indicates that the choice of 80 as a diagnostic titre is generally satisfactory from both a clinical and theoretical point of view.

The examination of serial samples of both serum and whey during the first three weeks after abortion show that the whey titres closely follow those of the serum though at a lower level. It is therefore probable that the major source of whey agglutinins is the blood and this suggestion is supported by the observation that titres of 320 or more were frequently recorded in samples which were apparently free of the organism. However, as the immunoglobulins in the serum and whey were not characterized it is not possible to provide a definite opinion on this point.

Sutherland & Berger (1944) suggested that the whey titre may be of value in the detection of faecal *Salmonella* carriers. They examined one cow twice and this had whey titres of 32 and 64. The animal was subsequently observed for a period of months by Rankin & Slavin (1947). By this time the whey titre had fallen to 10, despite the fact that the serum titres remained positive, and that faecal excretion was constant. A possible explanation for the findings in this case is that the cow had recently suffered an active, though possibly subclinical attack of salmonellosis, and that this was reflected in a transient rise in the whey agglutinin level.

Continued excretion of *S. heidelberg*, *S. dublin* and *S. paratyphi B* in milk has been recorded by Davies & Venn (1962), Weigt, Möller & Bleckmann (1972) and Thomas & Harbourne (1972) respectively. Thomas & Harbourne (1972) noted whey titres of O 80 and H 80–160 over a period of several weeks. Weigt *et al.* (1972) recorded a titre with an imprecise end-point of + + +/25, + +/50 and

+/100 in the milk of the affected quarter. They used the closely related organism *S. enteritidis* as the antigen, and as they gave no details of antigen production it is not possible to assess if this represents the true *S. dublin* titre or not. Nevertheless, these findings indicate that the whey test may be of some help in assisting the identification of cattle, which though thought to be very rare (Davies & Venn, 1962) do continually excrete *Salmonella* in milk. On the other hand the whey test is unlikely to be of much value in the retrospective identification of salmonella abortion cases.

The milk ring test

There have been a number of reports from Russia on the M.R.T. The most encouraging was that of Bodyagin & Leont'eva (1968) who found that the milk of cows immunized with an alum-adjuvant salmonella vaccine gave positive M.R.T. reactions even when the sample was diluted 1/124 in normal milk. Similarly the M.R.T. was still positive when bulk milk contained at least 8 % of milk from immunized cows. On the other hand Arkhangel'skii & Kartashova (1962) and Kurakina (1967) both record that there were often false positive reactions though Kartashova *et al.* (1969) found that the proportion of false positive reactions could be reduced, if the antigen was suspended in phenol-saline, instead of glycerin.

The small trial using the M.R.T. was disappointing. There were many false positives, even though the antigen was suspended in phenol-saline and, possibly even more important, there were some false negatives in wheys with high agglutinin titres.

Salmonella dublin *in the milk*

S. dublin was frequently cultured from the milk collected within a week of abortion though excretion had ceased by the end of the fourth week. This finding indicates that under no circumstances should unpasteurized milk be used for either calf feeding or human consumption for at least a month after the abortion.

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Isolations of subgenus III salmonellas (arizonas) in Cardiff, 1959–1971

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SUMMARY

Isolations of subgenus III salmonellas (arizonas) made in the regional Public Health Laboratory, Cardiff, between 1959 and 1971 are reviewed. The techniques of isolation are listed for the various materials examined. The necessity of using bismuth sulphite agar as a plating medium is stressed, as some strains might appear on brilliant green MacConkey agar as rapid lactose fermenters and be missed. The serotypes isolated in Cardiff are discussed with reference to isolations by other authors. The culture of a subgenus III salmonella from pig faeces is described. It is thought that this is the first record of such an isolation from a pig in the United Kingdom.

INTRODUCTION

Arizona species have not aroused much interest in Britain. This may be because they are rarely encountered except as a manifestation of imported infection (Edwards, Kauffmann & Stucki, 1960; Harvey & Price, 1962; Timms, 1971; Hughes *et al.* 1971). In this paper we record a series of isolations of strains of this subgenus made in the Regional Public Health Laboratory, Cardiff, in the period 1959–71.

MATERIALS

The samples examined for salmonellas were: (1) crushed bones imported from India and Pakistan, (2) crushed bone imported for fertilizers from Argentina, (3) abattoir drain swabs, (4) retail meat market drain swabs, (5) swabs of a tank in which terrapins were kept, (6) samples of caecal faeces and mesenteric glands from pigs slaughtered at Cardiff abattoir.

METHODS

Isolation methods varied with the materials examined and it will be simplest to present them in tabular form. This information is recorded in Table 1. These isolation techniques have been published in full elsewhere (Harvey, 1956; Harvey, Price & Dixon, 1966; Harvey & Price, 1967*a*, *b*; Harvey & Price, 1970; Kampel-

Table 1. *Methods of isolation of subgenus III salmonellas (arizonas)*

Material	Method			
	Pre-enrichment	Enrichment	Secondary enrichment	Plating media used
Crushed bones from India and Pakistan	Nutrient broth 24 hr. at 37° C.	Selenite F broth 24–72 hr. at 43° C.	Migration through soft agar at 37° C. with and without agglutinating sera	Wilson & Blair bismuth sulphite agar and brilliant green MacConkey incubated at 37° C.
Abbattoir and meat market drain swabs. Swabs from terrapin tank	Nil	Selenite F broth 24 hr. at 43° C.	Migration through soft agar at 37° C. with and without agglutinating sera	Wilson & Blair bismuth sulphite agar; brilliant green MacConkey agar; deoxycholate citrate agar
Samples from pigs slaughtered at Cardiff abbattoir	Nil	Selenite F broth 24 hr. at 43° C.; Kauffmann-Muller tetrathionate broth 24 hr at 43 °C.	Migration through soft agar at 37° C. without agglutinating sera	Brilliant green MacConkey agar; deoxycholate citrate agar
Crushed bone for fertilizer from Argentina	Nutrient broth 24 hr. at 37° C.	Selenite F broth 24 hr. at 43° C.	Migration through soft agar at 37° C. with and without agglutinating sera	Brilliant green MacConkey agar; deoxycholate citrate agar

Selenite F broth: Hobbs & Allison (1945). Kauffmann-Muller tetrathionate broth: Kampelmacher (1967), Heard, Jennet & Linton (1969). Wilson & Blair bismuth sulphite agar: de Loureiro (1942). Brilliant green MacConkey: Harvey (1956). Deoxycholate citrate agar: Leifson (1935), Hynes (1942). Secondary enrichment technique: Harvey & Price (1967*a*, *b*).

macher, 1967; Heard, Jennett & Linton, 1969). It will be noted that Kauffmann-Muller tetrathionate broth was used in the pig investigation in parallel with selenite F broth. This medium uses 0·018 M tetrathionate (Knox, Gell & Pollock, 1943) in comparison with 0·039 M tetrathionate employed in Rolfe's B formula (J. Morgan, personal communication, Rolfe, 1946). The medium with the lower concentration of the selective agent functions very well at 43° C. while that incorporating the 0·039 M concentration does not.

RESULTS

The results are shown in Table 2. The period of each separate investigation is recorded.

Some strains when first isolated from bone products were rapid lactose fermenters on brilliant green MacConkey agar, although they presented as typical salmonella-like colonies with surrounding metalic sheen on bismuth sulphite agar.

Table 2. *Subgenus III salmonellas isolated from different materials*

Material	No. of samples	Serotypes isolated
Crushed bone from India and Pakistan: 1959–1964	82	Ar 26:23:30 Ar 26:23:21 Ar 26:26:25 Ar 9a, 9c:29:31 Ar 16:22:31 Ar 20:24:28 Ar 29:24:31 Ar 29:33:21 Ar 30:23:31 Ar 30:27:28 Ar 9a, 9c:26:25
Abbatoir drain swabs: 1961–65	1641	Ar 26:29:30 Ar 26: – :30
Meat market drain swabs: 1967	446	Ar 26:32:21
Water from terrapin tank: 1967	1	Ar 26:23:30 Ar 24:24:28
Crushed bone from Argentina: 1968–9	170	Ar 30:22:31
Pig samples: 1968–71		
(*a*) Faeces	1796	Ar 26:29:30
(*b*) Mesenteric gland pools	132	Nil

On storage in the laboratory several of these strains failed to ferment lactose promptly on brilliant green MacConkey agar on re-examination and appeared as large green salmonella-like colonies after 24 hr. incubation at 37° C. The colonies produced by the strains found in abattoir and market drain swabs were indistinguishable from subgenus I salmonellas on both plating media. The two strains found in the terrapin tank sample differed in that serotype Ar 26:23:30 was a rapid lactose fermenter, both in lactose peptone water and on brilliant green MacConkey agar. In contrast, serotype Ar 24:24:28 presented as a salmonella-like colony on the neutral red indicator medium. Both strains were ONPG positive. The serotype isolated from the pig, Ar 26:29:30 had the same antigenic formula as some of the abattoir drain swab isolations and, like them, appeared on brilliant green MacConkey agar as colonies identical with subgenus I salmonellas. As this appears to be the first recorded finding of a subgenus III salmonella in a pig in the United Kingdom, the biochemical reactions of the strain are given in greater detail in Table 3.

The reactions recorded in Table 3 identified the strain as belonging to salmonella subgenus III, also known as the *Arizona* group. The organism, when cultured on a moist agar slope, readily agglutinated with *Salmonella* polyvalent H phase 1 and 2 serum, *Salmonella* polyvalent H phase 2 serum and *Salmonella* H phase 2 serum, factor 5. It did not agglutinate with *Salmonella* polyvalent O serum. Sera were provided by the Public Health Laboratory Service Standards Laboratory.

Cultures isolated up to 1962 were sent to Dr P. R. Edwards, Communicable Disease Center, U.S. Public Health Service, Atlanta, Georgia. Cultures isolated

Table 3. *Biochemical reactions of the* Arizona *strain isolated from pig faeces*

Dextrose	Acid and gas (1)	Citrate (Simmons)	+
Lactose, 1%	Acid and gas (2)	Potassium cyanide	−
Salicin	− (14)	Lysine decarboxylase	+
ONPG	+	Ornithine decarboxylase	+
Dulcitol	− (14)	Arginine dihydrolase	+
Gelatin liquefied		H_2S	+
Malonate utilized			

Figures in parentheses indicate days of incubation of tests.

subsequently were identified serologically at the Salmonella Reference Laboratory, London.

The pig serotype was identified as Ar 26:29:30 (Sa 61:k:1,5,7).

DISCUSSION

The serotype Ar 26:29:30 isolated from swabs placed in drains receiving material from slaughtered sheep and cattle and currently isolated from pig faeces has a close association with sheep. It has been found in sheep in several parts of the United States of America and also in Europe. There is a record of a previous isolation from a pig (Edwards, Fife & Ramsey, 1959). It has also been recovered from members of American red indian tribes in close contact with sheep (P. R. Edwards, personal communication).

Two of the eleven serotypes isolated from Indian and Pakistani crushed bone (Harvey & Price, 1962) had not been described before (Ar 20:24:28; Ar 26:26:25). Four of the serotypes (9a, 9c:29:31; 26:23:21; 26:23:30; 29:33:21) have since been found in Indian snakes (Kaura *et al.* 1972; I. P. Singh, personal communication). The serotype Ar 26:23:30, which we found in the terrapin tank, has also appeared repeatedly in monkeys affected with diarrhoea in which no shigellas or salmonellas were found (Edwards *et al.* 1959).

The strain 26:32:21, isolated in 1967 from the meat market drain swab, had been found in 1966 in a girl in Sheffield. This was the first isolation of a subgenus III salmonella from man in the United Kingdom. The source of infection was traced to a terrapin imported from Florida, U.S.A. (Plows, Fretwell & Parry, 1968). It is interesting that terrapins were on sale in the gallery of the Cardiff meat market.

From our own observations between the years 1959 and 1972, many of the strains isolated have been slow lactose fermenters. This may give a false picture of the situation. Some authors maintain that when cultures are selected without regard to lactose fermentation, the majority of *Arizona* strains recognized ferment lactose promptly (Solowey, 1947; Le Minor, Fife & Edwards, 1958). It is essential, if one is searching for arizonas to use a selective agar not dependent on lactose fermentation. In our experience, Wilson and Blair's bismuth sulphite agar, as modified by de Loureiro (1942), is very efficient in this connexion. Several strains found in Indian and Pakistani bones would have been missed had we relied entirely on brilliant green MacConkey agar. This brilliant green medium is, however, in our hands the plating medium of choice when subgenus I salmonallas

only are being considered (Harvey, 1956). For economy, it is undesirable to use multiple plating media. We do not employ bismuth sulphite agar in many of our routine investigations. Our examination of Indian crushed bone required the use of this medium and was somewhat in the nature of an academic exercise in the isolation of multiple serotypes. This prolonged study increased the chances of finding arizonas (Harvey & Price, 1967*b*).

We have little evidence that arizonas are of much importance in the United Kingdom. The recent introduction, however, of Ar 7a,7b:1,7,8:– in day-old turkey poults from California, U.S.A. (Joan Taylor, personal communication; Timms, 1971) and the record of serious disease in a Negro woman infected with a serotype of similar antigenic formula 7:1,7,8:– (Guckian, Byers & Perry, 1967) suggest that some attention should be paid to these rare invaders of Britain.

We are grateful to Dr B. Rowe of the Salmonella and Shigella Reference Laboratory, Central Public Health Laboratory, Colindale, London, and to Dr C. H. L. Howells, Director of the Regional Public Health Laboratory, Cardiff, for advice in the preparation of this paper. We are indebted to Dr R. Rohde of the National Salmonella Centre, Hamburg, who confirmed the antigenic structure of many of the strains.

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Fluctuations in the titre of antibody to a soluble antigen of myxoma virus in field populations of rabbits, *Oryctolagus cuniculus* (L.), in Australia

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SUMMARY

Results are presented on tests carried out over the past 5 years to measure the antibody titre of various rabbit populations to a soluble antigen of myxoma virus. In an unexpectedly high percentage of immune rabbits the antibody fell below measurable titres, and later rose to measurable titres without the advent of an observable epizootic of myxomatosis. The re-stimulation of the immune response is discussed in terms of re-infection and virus reactivation.

INTRODUCTION

Little is known about the relation of myxoma virus to the soluble antigens associated with its multiplication. Lesion material invariably contains soluble antigens, and at least eight have been demonstrated (Fenner & Ratcliffe, 1965). Animals with an active viraemia often have the soluble antigens circulating in their serum, and animals which recover from the disease have circulating antibodies to the soluble antigens. A technique for collecting blood samples on filter-paper strips and measuring the presence of soluble antigens or the antibodies to them was developed by Sobey, Conolly & Adams (1966, 1970).

For several years, this technique has been used to test field and laboratory populations of rabbits for the presence of soluble myxoma antigens and their antibodies. This paper presents some of the results obtained over the past few years in various studies.

MATERIALS AND METHODS

Serological technique

Five soluble antigens of myxoma virus have been detected in $(NH_4)_2SO_4$ precipitates (75% saturation) from rabbit lesion extracts (Reisner, Sobey & Conolly, 1963; Conolly & Sobey, unpublished). The precipitate was dissolved in 0·01 M potassium sulphate buffer, pH 7·0, and applied to a DEAE Sephadex (A-50) column. Elution was performed with a 0–0·5 M-NaCl linear gradient. Five separate

antigenic peaks, designated 'a' to 'e' respectively, were obtained. In all material prepared from 5-day lesions the 'd' antigen was most readily detected, and when run against recovery sera in two-dimensional gel diffusion plates always formed its precipitin band closest to the antigen source.

In the field, a spot of blood (obtained by puncturing the marginal ear vein of the wild rabbit with a lancet) was collected on a strip of filter paper. Care must be taken during the collection and storage of the blood samples to ensure that volatile substances, such as formalin, which denature proteins, are not allowed near them, otherwise the samples are spoilt. Any soluble antigens, or antibodies to them, in the blood were measured by their interference with the location of a precipitin band on an agar plate formed by the reaction between standard antigen and antibody solutions (Sobey *et al.* 1966, 1970). A rough estimate of the titre of the antibody in the blood sample was obtained by assessing the degree to which it interfered with the precipitin band when compared with a photograph of a $\log_2$ dilution series of a serum with a 1/128 titre. A grade 1 reaction was equivalent to a neat titre, a grade 2 reaction to 1/2, and grade 8 to 1/128. Zero on this scale indicates an absence or a low level of circulating anti-'d' antibody.

Management of enclosure

For one experiment, wild rabbits were maintained in a 0·5 acre enclosure. This was further surrounded by a rabbit-proof fence 6 ft. high, surmounted by electrified wires to prevent the entry of feral cats and foxes. Rabbits in the enclosure were examined every 3 weeks after being hand-captured in nets, or after being removed from their burrows. Adult females were palpated to see if they were pregnant and lactating, and the testes of males examined for size and position (either abdominal or scrotal). The size and location of any litters was noted; kittens were individually ear-tagged on reaching 3–4 weeks of age. Three blood samples were taken from each rabbit for serological examination.

Controlled-temperature experiment

One experiment involved exposing certain wild rabbits to various controlled temperatures. For this, use was made of a room in which the temperature could be maintained at any temperature from $-15°$ to $45° \pm 1°$ C. The humidity was unregulated and was not measured. The room housed 24 cages, each holding one rabbit. Water was supplied *ad libitum*, and food was given either as commercial pellets or green vegetables (cabbages, lucerne, etc.), or both.

Field experiments

The rabbit biology section of the Division of Wildlife Research, CSIRO has, for some 5 years, been intensively studying natural rabbit populations at three localities in New South Wales: Snowy Plains, in the subalpine region; Urana, in a Mediterranean-type climate; Canberra, on the slopes of the Dividing Range. These studies have involved the live-capture, tagging and watching of rabbits. As part of the routine, some rabbits which were live-trapped had a spot of blood taken for serological examination.

Viruses

Two cloned strains of virus were used in this work, Lausanne (Lu) and a field strain (FS/98) collected from the Canowindra district of New South Wales in 1967.

RESULTS

Controlled-temperature experiment

On 20 March, 19 of the 21 surviving immune wild rabbits from a previous experiment (Williams, in preparation) were placed in individual cages in the temperature-controlled room. For serological examination three blood samples from each rabbit were obtained at one time each week. The rabbits remained in the room for 7 months (10 March until 16 October), during which time, except for three periods, each of 3 weeks, the temperature was maintained at 60° F. (15·6° C.), and water and vegetables were provided *ad libitum*. During the three periods the animals were subjected to various stresses, including heat stress (twice), cold stress (once), nutritional stress (each time) and the administration of ACTH (first period only), to determine if any of these factors influence the titre of circulating antibody.

Fig. 1 shows the fluctuations in the titre of circulating antibodies in ten animals injected with 2·5 i.u. of ACTH during the first period of heat stress (1A), and in the remaining nine injected with 10 i.u. of ACTH (1B), during the 7 months of the experiment.

There were variations in the antibody titre of each rabbit, but it was difficult to correlate any particular variation with a particular treatment. In the great majority of cases the variations were not large. In only a few cases did the titre fall to non-detectable levels. In the case of rabbit no. 13, which has been fully discussed elsewhere (Williams, Dunsmore & Parer, 1972), the antibodies rose from an extremely low initial titre to a medium one following the development of the overt sign of myxomatosis during the first period of heat stress. Rabbit no. 13 showed signs of myxomatosis for 6 weeks (from 1 May until mid-June 1970) and soluble antigens were found in her blood during the second and third weeks in May. She was the only rabbit during this experiment which showed signs of myxomatosis.

Enclosure experiment

During September and October 1970 two litters were obtained from each of six does which had shown circulating antibodies to myxoma antigens. Kittens from eight of the litters were challenged when 3 or 10 weeks old with a field strain (FS/98) of myxoma virus, and the remaining kittens in four litters were challenged at comparable ages with the Lu strain of the virus. The animals were held in cages in an animal house from 3 weeks of age. Details of the number of rabbits involved are shown in Table 1. All 35 kittens challenged with the field strain survived. These recovered during December 1970 and January 1971. When recovered, 18 were placed into a 0·5 acre enclosure. None of the 18 Lu-challenged kittens survived.

The fluctuations in the antibody titre of the 18 recovered animals during 1971

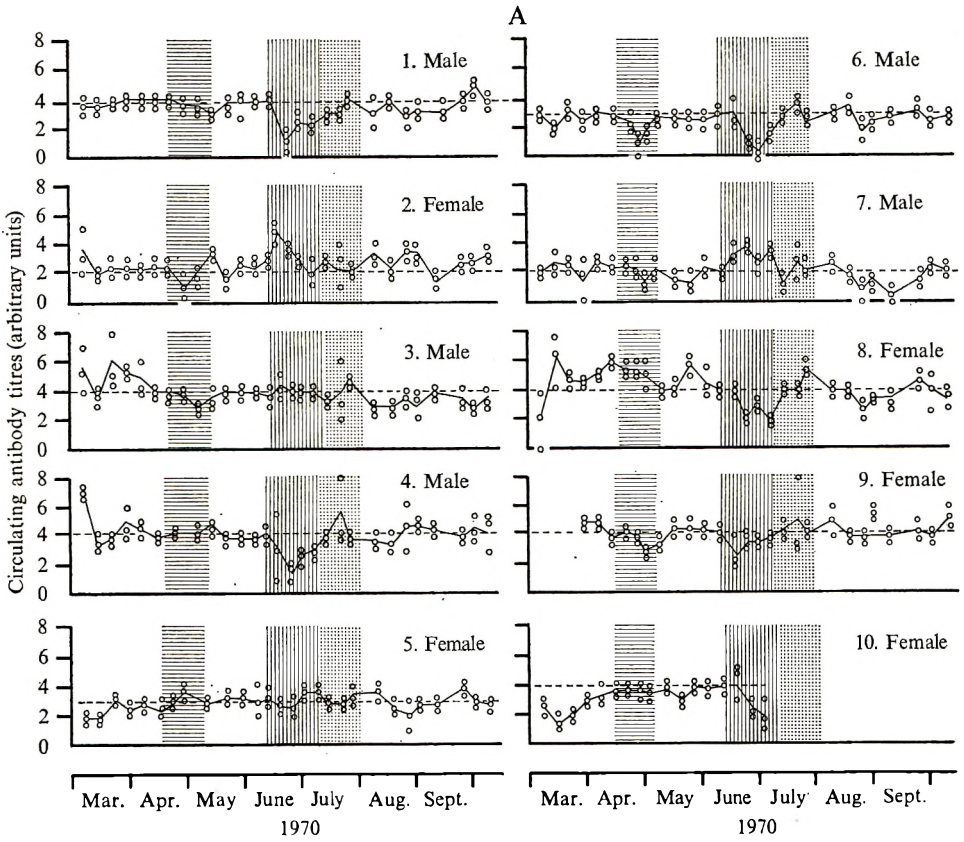

Fig. 1*a*. For legend see facing page.

Table 1. *Details of the number of young rabbits challenged at either* 3 *or* 10 *weeks of age with either a field strain or the Lausanne strain of myxoma virus*

	Age when challenged	
	3 weeks	10 weeks
Field strain		
No. challenged	19*	16†
No. survived	19	16
Lausanne strain		
No. challenged	11	7
No. survived	0	0
Mean survival time	12·0 days	13·0 days
Range	10–13 days	10–16 days

* Nine of the 3-week group and eight of the 10-week challenged group (†) either died on release into the enclosure following their recovery from myxomatosis or were used for other experiments.

and the early part of 1972 are shown in Fig. 2. Three of the ten rabbits (109, 136, 139) challenged at 3 weeks had a very low titre of antibody when released into the enclosure; all eight animals challenged at 10 weeks entered the enclosure with high antibody titres. From the time of being released most of the rabbits showed a decline in antibody titres until, by June 1971, all the rabbits challenged at 3 weeks and four of the eight challenged at 10 weeks had non-detectable antibody titres.

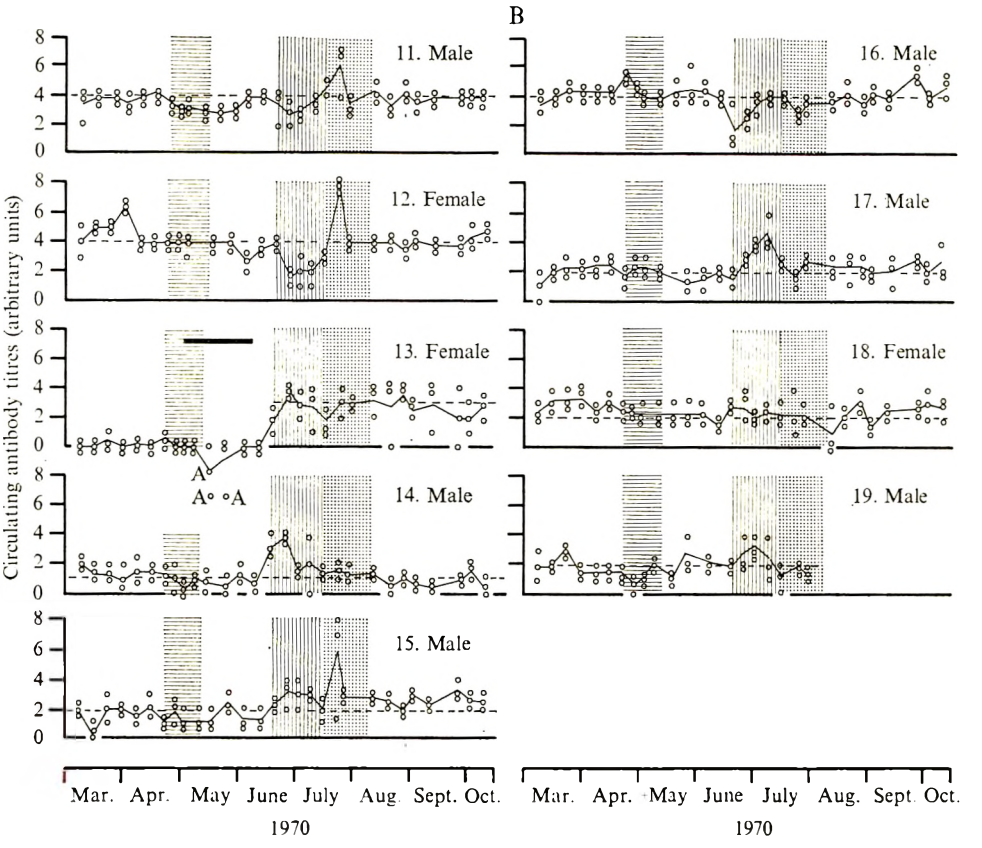

Fig. 1*b*

Temperature raised to 24°C, and vegetables withheld. Ten animals (*a*) received 10 i.u., and the remainder (*b*) 2·5 i.u. of ACTH daily.

Temperature dropped to 2°C, and vegetables withheld.

Temperature raised to 27°C, and straw only provided for food.

Fig. 1. The level of antibody to the 'd' soluble antigen of myxoma virus in 19 immune rabbits held in a constant temperature room from 10 March until 13 October, 1970. Temperature maintained at 16° C. and food (pellets and green vegetables) and water supplied *ad libitum*. The solid horizontal line denotes the period during which rabbit no. 13 was showing signs of myxomatosis and 'A' shows the presence of soluble antigens in her blood samples.

At the end of June the antibody titre of six rabbits (119, 132, 117, 128, 111, 112) rose sharply, whilst in the remainder of the animals it remained low. By late July 1971 the antibody titre of the former six animals had fallen, becoming undetectable in two cases (119, 132), and the other animals retained low antibody titres. In early August 1971 an outbreak of myxomatosis was first noticed amongst the young rabbits born in the enclosure; the last cases occurred in mid-September. Coinciding with the onset of myxomatosis the antibody titre of the adults rose, and remained high until the end of the epizootic.

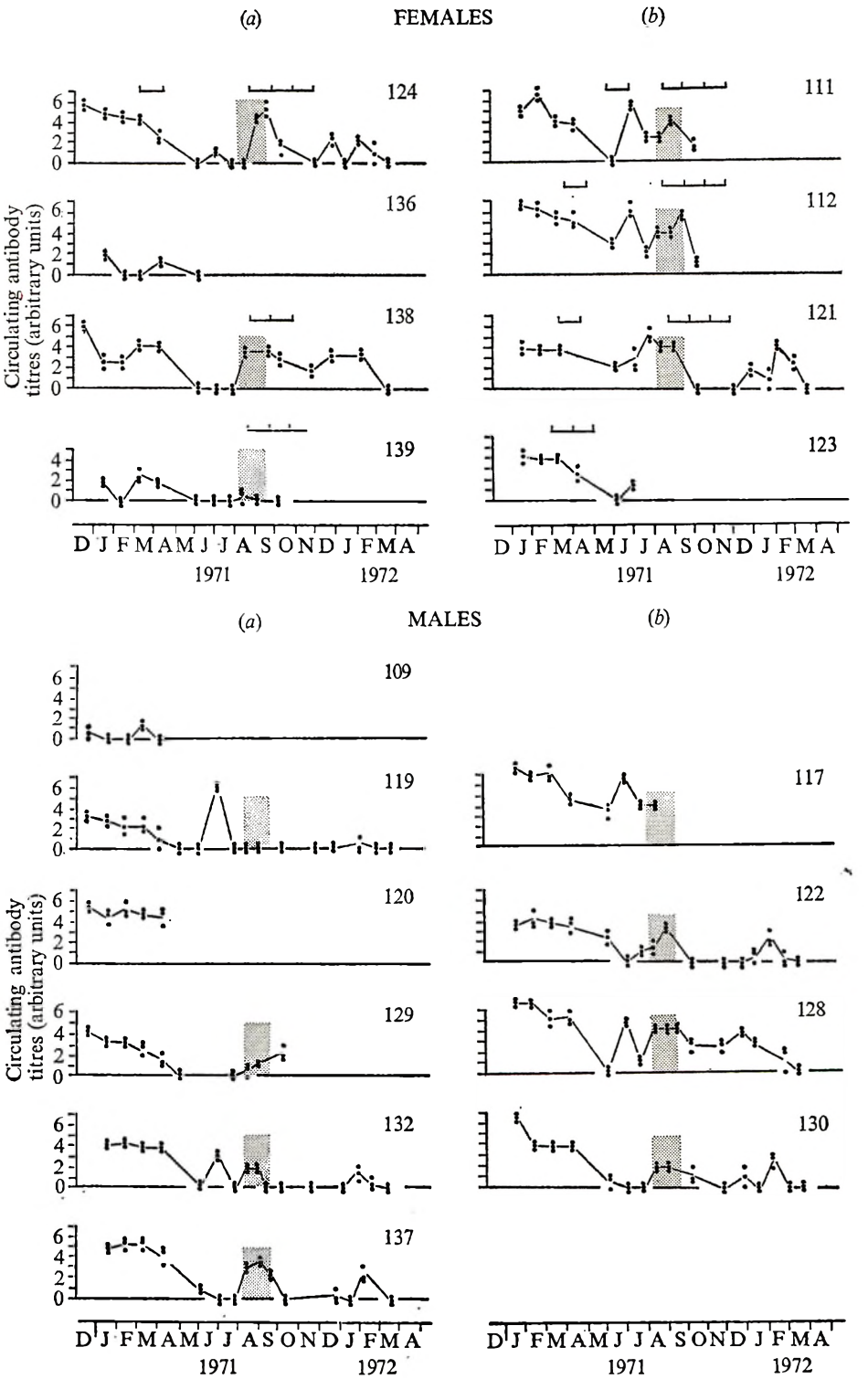

Fig. 2. The level of anti-'d' antibody in 18 rabbits which recovered from myxomatosis following challenge at either 3 (*a*) or 10 (*b*) weeks of age, which were maintained in a 0·5 acre enclosure from December 1970 until March 1972. The horizontal bars denote pregnancies in the females. The shaded area indicates the period of a myxomatosis outbreak which occurred amongst the young rabbits in the enclosure.

Following the epizootic, the antibody titre in all the animals again declined, and in many cases reached non-detectable levels during October and November. In mid-November four of the adults were killed by vandals. However, in the remaining adults the antibody titre rose during December and January, in the absence of any known cases of myxomatosis in the immediate vicinity (within a radius of 1 mile). The antibody titre declined during February, and by March 1972 was not detectable in any of the animals.

Field studies

Table 2 sets out the results obtained at three study sites maintained by the CSIRO Division of Wildlife Research. Table 2(*a*) shows, for two sites, the percentage of rabbits showing antibody in their blood each month which were known to have recovered from myxomatosis or to have lived through an epizootic and shown anti-'d' antibody. Table 2(*b*) shows the percentage of rabbits never seen to have been challenged with myxoma virus which showed antibody each month, from the three sites. The results obtained from each site are as follows:

Urana

The most complete record of fluctuations in the presence or absence of antibody each month in animals which had recovered from the disease was obtained at Urana. The myxomatosis history at this site from 1967 until 1972 has been fully described elsewhere (Williams & Parer, 1972). At Urana, myxomatosis is epizootic in November and early December of most years, transmission is usually rapid, and very few animals die of the disease. In addition, the morbidity rate is high, such that nearly all animals which survive an epizootic have been seen with the disease, or showed antibody in their blood. The main breeding season at Urana lasts from June until October (Parer, pers. comm.). At any time of the year, therefore, the rabbit population can consist of animals which have survived an epizootic and are immune and the young of the year which have not been exposed to the disease.

Table 2(*a*) shows that each year the lowest percentage of recovered animals with anti-'d' antibody was found in September, 1 month before the end of the breeding season and immediately before the annual epizootic. Over all the years, only 56% of the immune animals showed antibody in September. The percentage showing antibody increased through October, November, December and January each year, during the period when myxomatosis was active. By January each year, from 87 to 89% of the immune animals showed antibody. The percentage showing antibody declined through February and March, in which month only 67% of the immune animals showed antibody, In April and May a high percentage (93 and 97%) of immune animals showed antibody. From this high level the percentage of immune rabbits with antibody declined through the breeding season, to reach the low in September previously referred to. These monthly differences in the overall percentage of animals showing antibody are highly significant on a χ^2 test ($P < 0{\cdot}01$).

Table 2(*b*) shows that a very high percentage (17% overall) of the 688 young susceptible rabbits at Urana, in the only years tested (1967, 1968), showed anti-

Table 2. *The percentage of rabbits possessing anti-'d' antibody each month during various years from* 1967 *to* 1972 *at three sites*

(The figures in parentheses refer to the number in the sample each month.)

(*a*) Rabbits known to have recovered from myxomatosis, and/or to have possessed anti-'d' antibody following an epizootic

	J	F	M	A	M	J	J	A	S	O	N	D
						Urana, N.S.W.						
1967	—	—	—	—	—	—	—	—	—	—	71 (41)*	50 (18)
1968	—	71 (104)	24 (25)	—	—	76 (17)	100 (2)	83 (12)	0 (4)	33 (9)	54 (13)	89 (9)
1969	88 (32)	90 (10)	77 (102)	93 (30)	97 (30)	—	—	—	—	—	—	—
1970	—	—	—	—	—	81 (64)	97 (34)	85 (46)	64 (47)	73 (30)	92 (26)	94 (51)
1971	87 (75)	—	—	—	—	79 (48)	57 (110)	49 (85)	50 (30)	69 (13)	65 (57)	73 (56)
1972	89 (113)	—	—	—	—	—	—	—	—	—	—	—
Total	88 (220)	73 (114)	67 (127)	93 (30)	97 (30)	80 (129)	67 (146)	64 (143)	56 (81)	65 (52)	76 (127)	83 (134)
						Snowy Plains, N.S.W.						
1967	—	—	—	—	—	—	—	—	—	—	91 (23)	71 (14)
1968	100 (9)	100 (3)	50 (2)	100 (2)	0 (2)	—	—	88 (8)	80 (10)	50 (16)	9 (11)	50 (12)
1969	91 (11)	29 (7)	25 (4)	—	—	—	—	—	81 (26)	81 (16)	80 (5)	78 (9)
1970	100 (2)	—	67 (3)	100 (2)	—	0 (1)	100 (10)	—	100 (7)			
Total	95 (22)	50 (10)	44 (9)	100 (4)	0 (2)	0 (1)	100 (10)	88 (8)	84 (43)	66 (32)	67 (39)	66 (35)

(*b*) Rabbits known not to have been challenged with myxomatosis

						Urana, N.S.W.						
1967	—	—	—	—	—	—	—	—	—	—	8 (127)	13 (56)
1968	—	—	—	—	—	—	23 (31)	12 (154)	20 (167)	23 (62)	28 (87)	50 (4)
Total	—	—	—	—	—	—	23 (31)	12 (154)	20 (167)	23 (62)	16 (214)	15 (60)
						Snowy Plains, N.S.W.						
1967	—	—	—	—	—	—	—	—	—	—	24 (33)	6 (65)
1968	5 (114)	5 (61)	10 (41)	0 (26)	24 (21)	22 (9)	—	0 (33)	3 (38)	14 (64)	10 (176)	8 (237)
1969	4 (161)	3 (152)	5 (164)	9 (22)	—	—	—	—	0 (10)	23 (53)	2 (60)	1 (165)

Table 2 (*cont.*)

	J	F	M	A	M	J	J	A	S	O	N	D
1970	4	—	7	10	—	50	36	—	0			
	(72)		(57)	(40)		(2)	(36)		(9)			
Total	4	4	6	7	24	30	36	0	2	18	10	5
	(347)	(213)	(262)	(88)	(21)	(11)	(36)	(33)	(57)	(117)	(93)	(467)
						Canberra, A.C.T.						
1967	—	—	—	—	—	—	—	—	—	0	2	0
										(45)	(50)	(4)
1968	0	0	—	—	—	—	0	6	3	6	2	2
	(20)	(5)					(5)	(50)	(38)	(96)	(113)	(51)
1969	—	13	0	2	0	0	0					
		(105)	(75)	(85)	(8)	(79)	(11)					
Total	0	13	0	2	0	0	0	6	3	4	2	2
	(20)	(110)	(75)	(85)	(8)	(79)	(16)	(50)	(38)	(141)	(163)	(55)

body. It should be remembered, however, that virtually all the animals tested were under 10 weeks of age, and the possibility exists that many of the kittens possessed maternal antibodies (Fenner & Marshall, 1954).

Snowy Plains

In contrast to the situation at Urana, myxomatosis at Snowy Plains does not appear to occur annually. Details of the myxomatosis history at Snowy Plains from 1967 until 1972 have been set out elsewhere (Dunsmore, Williams & Price, 1971; Dunsmore & Price, 1972). Briefly, an outbreak of the disease was in progress when the study began in March 1967, and all the animals entering the 1967 breeding season, which at Snowy Plains lasts only from October to December each year, were survivors of the outbreak. The next apparent outbreak of myxomatosis at this site was in the winter of 1969, and lasted from March until August, affecting susceptible animals born in 1967 and 1968. Few animals (a maximum of 14%) recovered from the disease, and fewer still (4% of the susceptible animals) avoided infection. Minor epizootics, affecting only a few animals, occurred in 1970 and 1971. The next major epizootic occurred in the summer of 1972, affecting animals born in 1969, 1970 and 1971. In this outbreak, recovery was much higher than in 1969, being about 67%.

Table 2(*a*) shows that the overall fluctuations in the percentage of immune animals showing antibody at Snowy Plains from 1967 to 1970 were similar to those obtained at Urana. Thus, a low percentage of immune animals (66%) showed antibody during the breeding period (October–December), increasing to 95% in January, and declining through February and March (50 and 44%). A total of only seven immune animals was trapped in April, May and June during the study period, which is an insufficient number to provide accurate information on antibody fluctuations. However, as at Urana, in the months immediately before the breeding season (July–September at Snowy Plains), there was a high but declining number of immune animals with antibody (100, 88 and 84%).

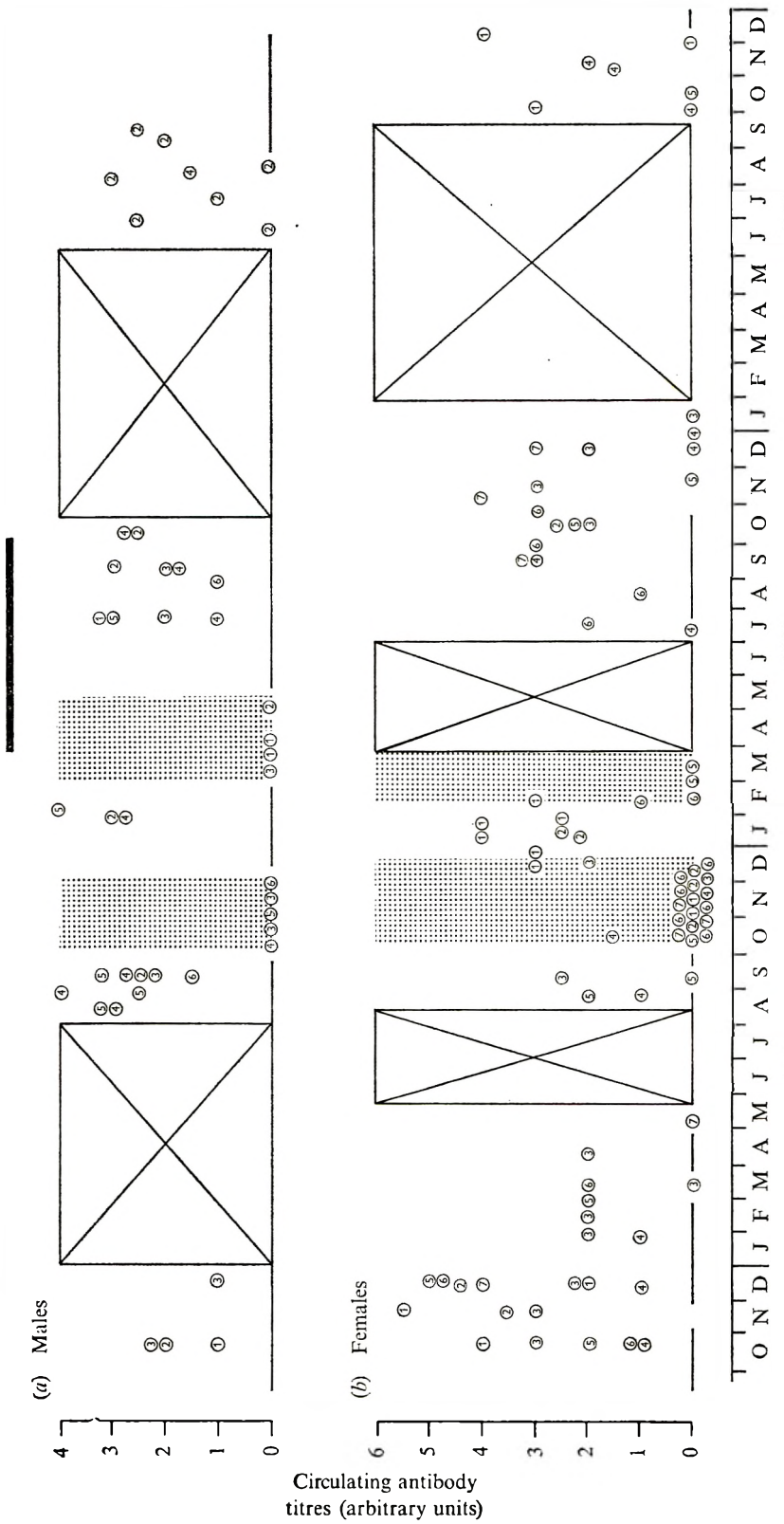

Fig. 3. The level of anti-'d' antibody in six male (*a*) and seven female (*b*) rabbits at Snowy Plains, New South Wales, which recovered from myxomatosis in March 1967, from October 1967 until December 1970. The shaded areas indicate the periods when antibodies decreased to non-detectable levels. The crossed-out portion shows the periods during which the rabbits were very difficult to trap. The solid horizontal line denotes the period of an epizootic of myxomatosis which occurred amongst the population.

Unlike Urana, several animals at Snowy Plains were trapped sufficiently frequently to provide data on antibody titres in individual rabbits from 1967 to 1969. The results obtained from six male (Fig. 3*a*) and seven female (Fig. 3*b*) rabbits are shown in Fig. 3. This confirms that the fluctuations in antibodies demonstrated by the monthly samples at Snowy Plains are actually occurring in the case of individual rabbits.

Table 2(*b*) shows that, overall, 7% of the susceptible animals at Snowy Plains showed antibody. During the breeding season (October and November) many young rabbits (18 and 10%) showed antibody. In these cases, the antibody might have been derived from the mother. However, a very large proportion (from 24 to 36%) of the susceptible rabbits tested in May, June and July at Snowy Plains showed antibody. Since these animals would have been at least 6 months old, the antibody could not be of maternal origin (Fenner & Marshall, 1954).

Canberra

The history of myxomatosis in the Canberra population from 1966 until 1969 (Williams, Fullagar, Davey & Kogon, 1972; Williams, Fullagar, Kogon & Davey, 1973), is similar to that described for Snowy Plains. The population was started in mid-1966 with 36 adult rabbits, five of which had previously recovered from myxomatosis. None of the five immune rabbits survived past 1966. The rabbits bred in 1966 (September–January), 1967 (August–November), 1968 (July–December) and 1969 (March–April). An outbreak of myxomatosis occurred in the winter months (July–September) of 1969, as a result of which only two kittens, which avoided infection, survived.

Thus, in 1967, 1968 and 1969, when the sampling for antibodies was carried out, there were no immune animals in the population. Table 2(*b*) shows that of the 840 samples of Canberra rabbits tested, only 4% showed antibody. Further, all of these were scored as 1, which is approaching the limits of detectability of the antibody.

DISCUSSION

The results show that in field populations of rabbits the titre of antibody to the 'd' soluble antigen of myxoma virus varies in time after recovery from infection; at times it may fall below the level of sensitivity of the measurement technique. Most animals in this study had recovered from an attenuated ('field') strain of myxoma virus. Their chances of recovery had been increased by high temperatures (as at Snowy Plains in the summer of 1967), assisted by maternal antibodies (as with the enclosure rabbits) and genetic resistance (as at Urana each year). However, where field strains operate at low temperatures (i.e. in winter), amongst rabbits in which the genetic resistance is low, and maternal antibodies are absent, the chances of recovery are low (Dunsmore *et al.* 1971; Williams, Fullagar *et al.* 1972, Williams *et al.* 1973).

In many of the recovered animals the antibody titre decreased with time, and often fell below the threshold of measurement. It has often been observed that antibodies to a wide variety of antigens decline to low levels after a period following

the initial challenge (Boyd, 1956). This has also been shown with the complement-fixing antibodies of myxoma virus in rabbits (Fenner, Marshall & Woodroofe, 1953). Sobey (unpublished) has observed a decline in the anti-'d' antibodies in recovered laboratory rabbits. In all these cases the antibodies to the various attenuated myxoma virus antigens had fallen to low titres within 6 months, which agrees reasonably closely with the declines observed in the field populations in this study.

The observation that high antibody titres may reappear after they have fallen very low suggests some form of stimulation. It seems likely that such stimulation is due to re-exposure to the 'd' antigen, which, since it is probably a by-product of virus multiplication, suggests that virus had multiplied in the animal. Virus can multiply in recovered animals, for a short time at least, if the animal is exposed to virus some months after recovery (Fenner *et al.* 1953). Stimulation of the anti-'d' antibodies may thus occur as a result of re-infection. Such is the probable explanation for the rise in antibody titre in the enclosed population at Canberra in August/September 1971, since myxoma virus was active amongst the susceptible animals in the enclosure, and the immune adults would have continually been exposed to the virus.

There are two possible explanations for the rise in antibody titres which, in the absence of any obvious virus activity, occurred in the populations in the enclosure and at Snowy Plains in summer, and at Urana in autumn. Firstly it is possible that the stimulation was caused by re-infection with a strain of myxoma virus which was so attenuated as to escape detection. This seems unlikely, because myxoma virus relies on mechanical transmission by arthropods from skin lesions for dissemmination; if the skin lesions were so slight as to escape intensive observation, the possibility of transmission would be low.

An alternative explanation is suggested by recent evidence (Williams, Dunsmore & Parer, 1972) that myxoma virus may survive in a latent phase. Latency is probably the result of a dynamic balance forming between the immune mechanism of the animal and the infecting virus. If the immune response falls below a certain level, the virus may multiply, re-activating the 'immunological memory', restoring the balance. It may be postulated that in most cases multiplication of virus after re-activation results in a subclinical infection. In a few cases, however, virus multiplication after reactivation may result in overt infection. This may be most likely to occur when the animal is under stress. Some other latent viruses, including *Herpes simplex* (Rasmussen, Marsh & Brill, 1957), *H. zoster* (Hope-Simpson, 1965), rabies (Soave, 1964) and psittacosis (Meyer, 1942), appear in various animals under a variety of stressful conditions. It seems likely that this stress-induced reactivation of virus leading to overt disease also applies to myxomatosis (Williams, Dunsmore & Parer, 1972).

There is evidence which supports this hypothesis. The times when animals in field populations have low antibody titres, and when presumably reactivation and multiplication of latent myxoma virus is more probable, are late spring and early autumn. These are both times when outbreaks of myxomatosis have been noted to start in field populations, resulting in either summer or winter epizootics.

Additionally, in such summer and winter epizootics which have been studied in detail (Williams & Parer, 1972; Dunsmore *et al.* 1971; Williams, Fullagar *et al.* 1972, 1973) the re-activation of latent virus resulting in overt infection in certain rabbits was considered to be the source of virus initiating the epizootics. In these cases, climatic conditions and population density were such that they could be regarded as stressful in the periods immediately before the epizootics. In the same populations in intervening years when population densities were lower, no myxomatosis occurred.

Accepting this hypothesis, there remains unexplained the reason for the appearance of antibodies in a substantial percentage of the susceptible rabbits in the autumn at Snowy Plains. If the possibility that a highly attenuated strain of myxoma virus was present in the population is discounted, there remains the explanation that reactivation of latent virus occurred. If the latter is correct, it means that latent myxoma virus can pass from parent to offspring. A similar hypothesis was advanced, on other circumstantial evidence, to explain the appearance of myxoma virus in completely susceptible rabbit populations in which winter epizootics occurred (Williams, Fullagar *et al.* 1972, 1973).

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Antigenic analysis of prototype influenza A (H3N2) strains by the antiserum absorption method

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SUMMARY

Prototype strains of the influenza A (H3N2) virus can be arranged on a gradient showing the degree of the antigenic drift which the haemagglutinins of the strains have undergone. The demonstration of fine antigenic differences is based on an antiserum absorption test which allows a detailed antigenic analysis of strains. The gradient provides information on variation in strains occurring in different geographical areas and its use may be helpful in differentiating between introduced strains and locally developing variants.

INTRODUCTION

The differences between haemagglutinins of influenza virus strains are usually investigated by the cross haemagglutination-inhibition (HI) test. In general, the more time that has elapsed between the isolation of two strains of the same subtype the more pronounced antigenic difference is demonstrable between them, suggesting that the antigenic drift of the influenza virus is, in general, progressive. However, owing to different degrees of immunity of the populations in which different strains circulate, it may occur that strains isolated at the same time in different geographical regions are at different stages of progression or even a later strain may be at a less advanced stage. The cross HI test provides little information in this respect, especially late in the interpandemic period. Since, supposedly, the furthest progressed strains are of greater importance as regards future epidemics, the investigation of influenza virus strains from this point of view needs more sensitive tests. Such a test may be used in selecting the best strains for vaccine production.

In the present study several prototype strains of influenza A (H3N2) virus were investigated by an antiserum absorption test to determine their proper place on the gradient of antigenic drift. The method in general was described long ago (Takátsy & Fürész, 1954; Takátsy & Fürész, 1957; Takátsy & Barb, 1958) and has been somewhat modified for the present purpose.

MATERIALS AND METHODS

Influenza A (H3N2) virus strains

International prototype strains Hong Kong/1/68, England/878/69, Hong Kong/-107/71 and England/42/72 and strains Hungary/1/69, Hungary/20/70, Hungary/-1/71, Hungary/92/71 isolated by us.

Preparation of antisera

Roosters were immunized intravenously with a purified virus suspension containing 3000 haemagglutination (HA) units per ml. Three 1 ml doses were administered at weekly intervals. Six to 8 weeks later a 1 ml. booster dose was given. The birds were exsanguinated 2 weeks after the last injection. The blood, taken with sodium citrate, was centrifuged and the plasma was inactivated at 56° C. for 30 min., then re-centrifuged. The supernatant plasma, with 0·05 % sodium azide as preservative, was used as antiserum.

Purified concentrated virus

Allantoic fluid containing virus was mixed with a 2 % suspension of formalized chicken erythrocytes. The mixture was kept at 4° C. for 30 min. while shaken at intervals. Finally, the virus was eluted from the sedimented erythrocytes in 5 % NaCl at 37–40° C. The supernatant was dialysed against distilled water for 24–36 hr. By the end of this period the NaCl concentration in the inner fluid had decreased to between 0·2 and 0·3 % and, consequently, the virus formed a precipitate, which was then sedimented by centrifugation at 3000 rev./min. The sediment was resuspended in phosphate-buffered saline (PBS) to obtain a virus suspension with a titre of approximately 10^5 HA units/ml. After centrifuging at 3000 rev./min. the supernatant of this suspension was used as purified concentrated virus (stock suspension).

HA and HI tests

Were carried out in the Microtitrator* (Takátsy, 1955) apparatus.

Antiserum absorption test

Serial twofold virus dilutions were prepared from each stock suspension in two rows, using the 0·05 ml. Microtitrator loops. In the first well of the second row the dilution was 1/3 (one loopful in 0·1 ml diluent); in all the other wells the volume of the diluent was 0·05 ml. After the dilution series have been completed, a loopful was removed with the 0·05 ml. loop from the first well of the second row. Thus, by combining the dilutions in the two rows, a series consisting of 12 dilutions (1/2, 1/3, 1/4, 1/6, *etc.*) was obtained. Subsequently, 0·05 ml. of the serum to be absorbed and 0·05 ml. PBS were added to each dilution. The contents of the wells were mixed up with a rust-free wire, beginning at the highest virus dilution, and the titration trays were incubated at 37° C. for 30 min. Meanwhile the trays were shaken several times either gently mechanically or by a suitable vibrator.

After incubation a sample of 0·025 ml. was taken from each mixture for HA test.

* (R) Labor MIM, Budapest.

Table 1. *Data from a representative antiserum absorption test*

	Virus Hung/1/71						
Virus dilution	1/2	1/3	1/4	1/6	1/8	1/12	1/16
Ha units per diluted sample	10^5	$6{\cdot}6 \times 10^4$	$5{\cdot}0 \times 10^4$	$3{\cdot}3 \times 10^4$	$2{\cdot}5 \times 10^4$	$1{\cdot}6 \times 10^4$	$1{\cdot}2 \times 10^4$
	Hung/92/71 serum absorbed with the above indicated virus dilution						
Residual Ha units per sample	3000	500	27	—	—	—	—
HI titre to:							
Hung/92/71	N.T.	N.T.	N.T.	64	96	256	512
Hung/1/71	N.T.	N.T.	N.T.	< 2	8	32	128
HK/1/68	N.T.	N.T.	N.T.	32	64	128	384
Eng/878/69	N.T.	N.T.	N.T.	< 2	6	24	64

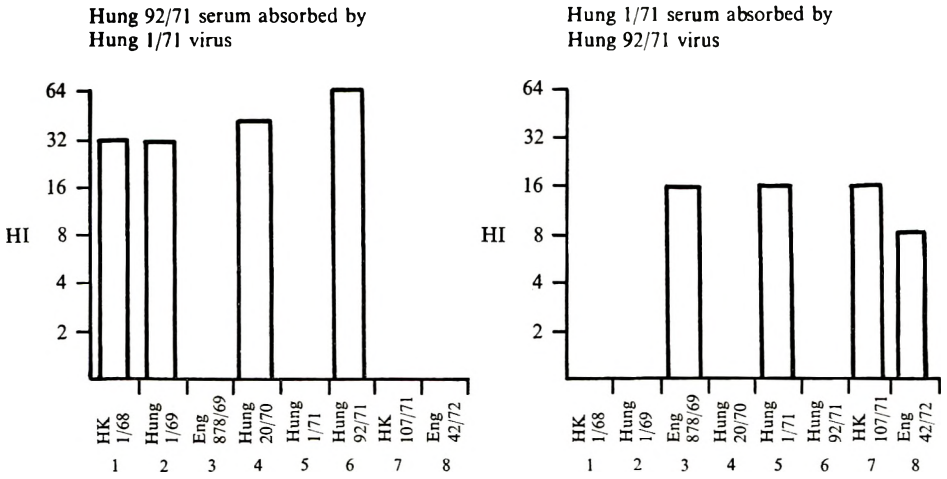

Fig. 1. Results of cross-absorption tests between influenza A strains Hung/92/71 and Hung/1/71

With the serum–virus mixtures containing no haemagglutinating virus, HI tests were carried out, using different virus strains. A representative serum-absorption test is shown in Table 1. In this experiment the 1/6 dilution was the first dilution showing no haemagglutination after serum absorption. In the same sample no HI antibody to the strain used for adsorption (Hung/1/71) could be detected, whereas the titre of the residual homologous antibody (to strain Hung/92/71) was 1/64, i.e. at least 32 times higher. The corresponding ratios from the tests carried out with serum samples obtained by adsorption with 1/8, 1/12 and 1/16 diluted virus suspensions were much lower: 12, 8 and 4, respectively, indicating that the absorption with the 1/6 diluted virus produced the most sensitive mixture for strain differentiation by simple HI titration. On the basis of such experiments, we call the first serum–virus mixture in which the HA test is negative optimally absorbed serum. For further strain differentiation larger volumes of absorbed samples of the optimal relative composition were produced.

RESULTS

The antiserum absorption test as carried out according to the Microtitrator system has the great advantage that (i) very small volumes of concentrated virus are needed for the test and (ii) the results are independent of the virus and serum titre, i.e. standardized preparations are not needed.

Our method enabled us to follow the antigenic drift of the haemagglutinin of the influenza A (H3N2) virus by the analysis of the residual antibody in antisera optimally absorbed with different strains of the same main antigenic composition. We attempted to establish a gradient by arranging strains with those showing the most antigenic drift given the highest rank. Ranking of the strains has been based on our finding that if a serum is optimally absorbed with a strain standing higher on the gradient than that to which the serum is homologous the residual antibody

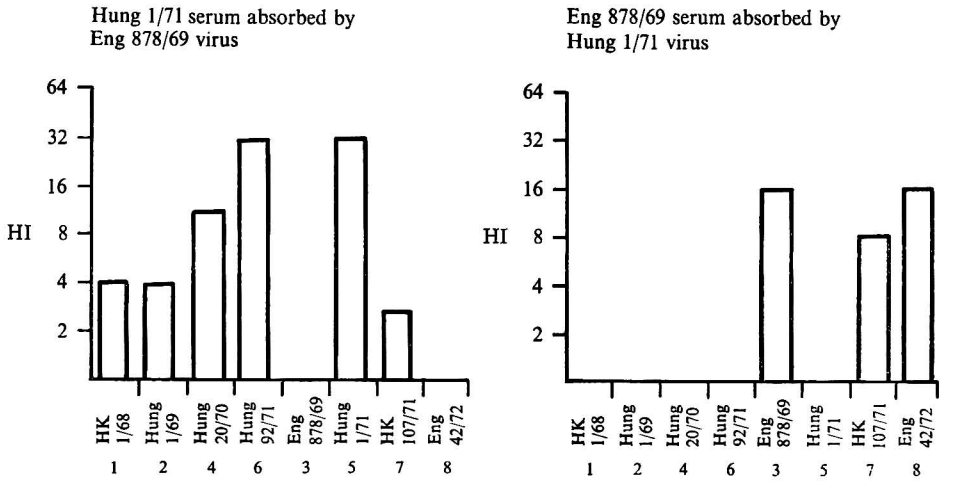

Fig. 2. Results of cross-absorption tests between influenza A strains England/878/69 and Hung/1/71

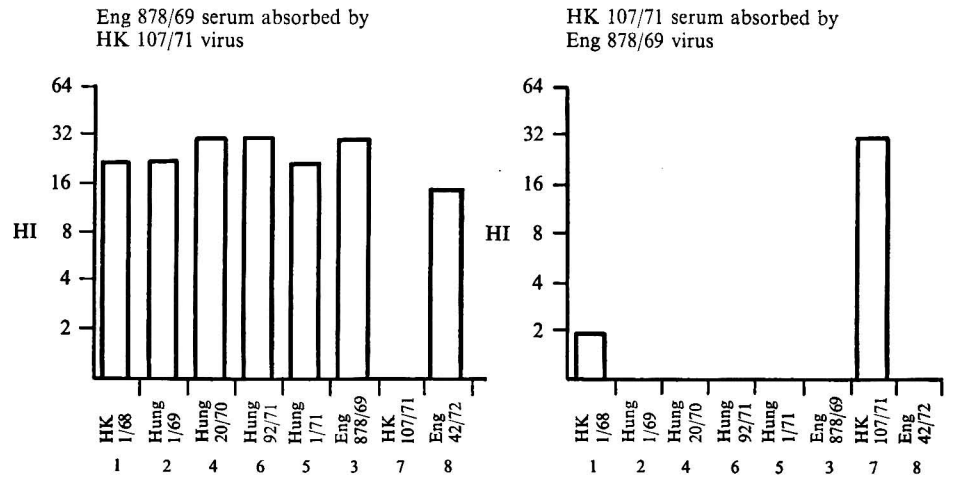

Fig. 3. Results of cross-absorption tests between influenza A strains England/878/69 and Hong Kong/107/71.

will react with the homologous strain, and also with all those standing lower on the gradient, but not with those standing higher and vice versa.

This principle is demonstrated in Fig. 1, illustrating the cross-absorption tests between strains Hung/92/71 and Hung/1/71. The optimally absorbed sera were examined with all the eight strains under study. The order of the strains in Figure 1 agrees with the chronological order of their isolation. According to the above principle, strain Hung/1/71 stands higher on the gradient than strain Hung/92/71, showing that its antigenic drift is more progressed. Even the England/-878/69 strain should be placed higher than Hung/92/71.

The experiment shown in Figure 1 gives no information as to which of strains England/878/69 and Hung/1/71 stands higher on the gradient. In a further

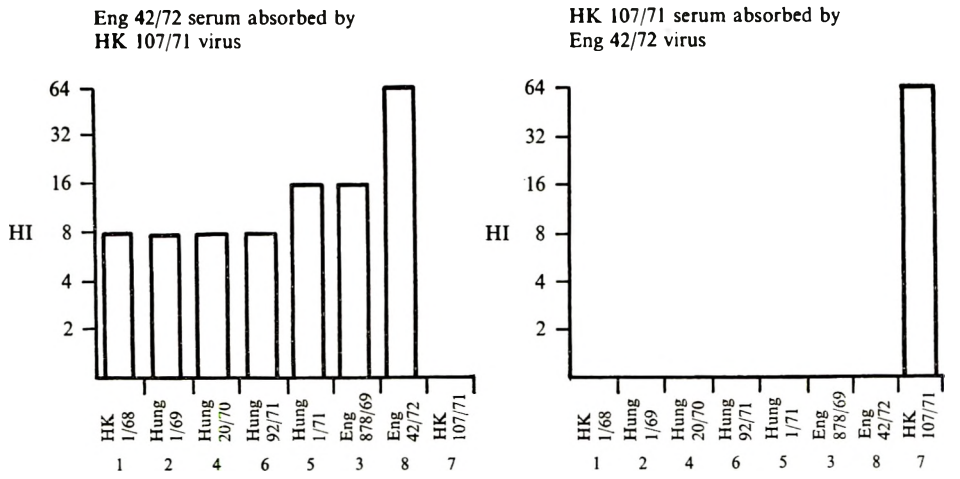

Fig. 4. Results of cross-absorption tests between influenza A strains England/42/72 and Hong Kong/107/71.

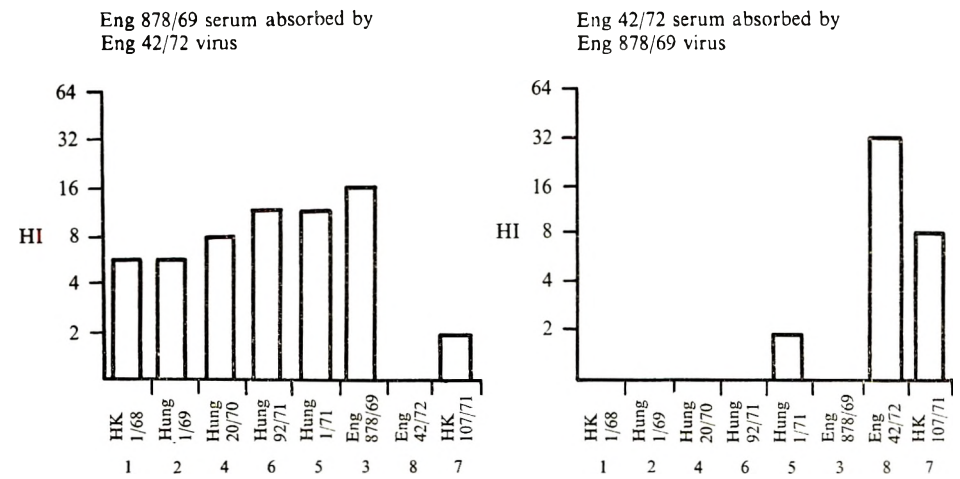

Fig. 5. Results of cross-absorption tests between influenza A strains England/878/69 and England/42/72.

experiment (Fig. 2) these two strains were subjected to the cross-absorption test. It is clear that England/878/69 stands higher. In the left graph of Fig. 2 the clear pattern is somewhat disturbed by the positive reaction of the residual antibody in serum Hung/1/71 with strain Hong Kong/107/71; the graph on the right side, however, clearly shows that the antigenic drift of the latter strain is more progressive. We return to this phenomenon in the Discussion.

Since the order of the Hungarian strains on the gradient had been established and it was proved by the experiments illustrated in Fig. 3, the relation to each other of the most progressive strains England/878/69, Hong Kong/107/71 and England/42/72 was examined (Figs. 3–5). Hong Kong/107/71 has proved to be the most progressive strain, followed by England/42/72 and England/878/69.

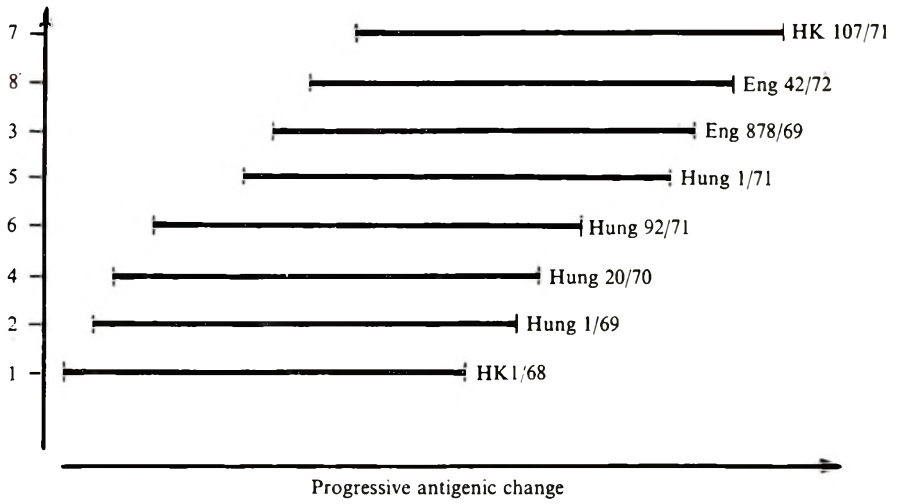

Fig. 6. Diagram showing the gradient from least to greatest antigenic drift of the eight strains of influenza A virus.

Accordingly, the gradient from least to greatest drift is as follows: Hong Kong/-1/68, Hung/1/69, Hung/20/70, Hung/92/71, Hung/1/71, England/878/69, England/-42/72 and Hong Kong/107/71 (Fig. 6).

DISCUSSION

The antigenic variation of the inter-pandemic antigen drift of human influenza A virus may be characterized as a step-by-step progressive change (Takátsy & Fürész, 1954; Takátsy & Fürész, 1957; Takátsy & Hamar, 1955) probably originating from spontaneous mutations. The selection of the mutants is governed by the population immunity. The role of antibodies in this process was proved in model experiments by Archetti & Horsfall (1950) and recently by Laver & Webster (1968), who characterized the antigenic drift by well-defined changes in the 'peptide map' of the virus. Since, if the propagation of influenza virus in the human population is not hindered by other factors, the antigenically most progressed strains have the greatest chance of causing the next epidemics, determination of the degree of this progress is of epidemiological importance. This character of the strains should be taken into consideration when strains for vaccine production are selected. The orientation in this field is greatly facilitated by the antiserum absorption test described in this paper.

Furthermore, it is clear from the present results that the anti-haemagglutinin molecules induced by any of the influenza A virus strains are heterogeneous. The antibodies demonstrable in an antiserum after absorption with different heterologous strains are sharply different from each other. It is not clear, however, whether these heterogeneous antibodies are induced by a heterogeneity of the haemagglutinins on the same strain or are a result of the heterogeneity of the immune response to the same antigen. We prefer the first alternative.

The antigenic drift of the haemagglutinin may be explained by serial spon-

taneous mutations. Similar mutants may arise and cause epidemics in different geographical areas and, if the population immunity in the different regions is similar, the antigenic drift may progress in parallel in remote countries. In some areas, however, more progressive mutation may occur than elsewhere and thus a strain may arise which has the greatest chance to cause an epidemic. In our opinion England/878/69, England/42/72 and Hong Kong/107/71 are such strains which called attention of the investigators using the cross HI test. The fact that in 1971 we isolated strains closely related to England/878/69 does not indicate that this strain had been introduced to Hungary. Since the Hungarian strains stand lower than England/878/69 on the gradient of antigenic drift, we suppose that they had developed independently and not by regression from a more progressed strain.

The Hong Kong/107/71 serum as absorbed by the less progressed England/-878/69 virus did contain some antibody inhibiting the prototype strain Hong Kong/1/68. It is of interest that this single irregularity in our scheme occurred in relation to two strains isolated at different times in the same area.

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Typhoid fever imported from Mexico to Switzerland. Studies on R factor mediated chloramphenicol resistance

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SUMMARY

A case of typhoid fever caused by *Salmonella typhi* occurred in Geneva. The patient was probably infected in Mexico City. The strain isolated from this patient corresponds with the description of the Mexican *S. typhi* strain, since it is a degraded Vi-strain resistant to chloramphenicol, streptomycin, sulphonamides and tetracyclines. It carried an *fi*$^-$ transferable R factor with a CSSuT resistance pattern. It can be accepted that this case forms part of the Mexican outbreak of chloramphenicol-resistant typhoid fever which has already been observed in visitors to Mexico from England and the United States.

INTRODUCTION

The fact that drug resistance can be transferred between enterobacterial strains by conjugation has important epidemiological and clinical implications. Although resistance transfer apparently occurs less frequently *in vivo* than *in vitro*, it has been demonstrated in man and animals (reviews: Watanabe, 1963; Anderson, 1968). Clinical isolates capable of transferring multiple drug resistance to other strains during mixed cultivation have been described in epidemic shigellosis (Ochiai, Yamanaka, Kimura & Sawada, 1959), in urinary tract infections (Smith & Armour, 1966), in nosocomial infections (Gardner & Smith, 1969) and in *Salmonella* infections (Anderson & Lewis, 1965*a*, *b*; Gill & Hook, 1966).

Recent reports from Mexico have described a protracted outbreak of typhoid fever caused by a strain of *Salmonella typhi* resistant to chloramphenicol because it carries a transferable resistance factor (W.H.O. Weekly Epidemiological Record, 1972). The recent account by Anderson & Smith (1972) of typhoid infection in two British visitors to Mexico, and the occurrence of a similar case at our hospital in a patient returning from Mexico City, prompted us to investigate the resistance pattern of the strain of *S. typhi* isolated from this case and to document its identity with the Mexican strain.

CASE REPORT

A 22-year-old girl was admitted to the Hospital on 6 June 1972 with a 6 days' history of fever, cough, nausea and vomiting. She had been perfectly well until 6 weeks before admission, when she visited South and Central America. While in Mexico City, 3 weeks before her admission to hospital in Switzerland, she suffered nausea, vomiting and diarrhoea of 24 hr. duration, for which she received a short course of chloramphenicol. The symptoms abated until her return to Switzerland. Six days before admission she developed fever up to 40° C., anorexia, nausea and vomiting, followed by an unproductive cough.

On admission the physical signs included a temperature of 40° C., a heart rate of 88/min., a short atypical systolic murmur and a slightly tender abdomen without peritoneal signs.

Routine laboratory findings included a hematocrit reading of 45%, a white blood cell count of 4000/mm.^{3} with a slight shift to the left, a blood urea nitrogen of 14 mg./100 ml., normal electrolytes and normal liver function tests. All blood cultures and two stool cultures taken on the first 2 days after admission yielded *S. typhi* resistant to chloramphenicol but sensitive to ampicillin by the standard disk method.

Treatment was instituted on the 3rd hospital day with 4 g. of ampicillin by mouth, and the patient recovered. Antibiotic treatment was continued for 3 weeks. The TO and TH agglutinin titres were 1/400 and 1/200 respectively on admission; they rose to 1/800 and 1/400 after 1 week and had returned to 1/100 and 1/50 1 month later. However, no significant Vi agglutinin titre was detected during or after the first treatment period.

Because of intermittently positive stool cultures for *S. typhi* during the 2 weeks after completion of the antibiotic treatment, a second 6 weeks' course of ampicillin was given. Careful monitoring of the stools showed all specimens tested to be negative for *S. typhi*; they have remained negative since then.

LABORATORY STUDIES

Bacteriology. The resistance pattern of 9 isolates of *S. typhi* (7 from stools and 2 from blood cultures) was studied, using the standard disk diffusion method. All were resistant to chloramphenicol and belonged to a degraded Vi-strain.

Minimal inhibitory concentrations (MIC). Small inocula (0·1 ml. of an overnight broth culture diluted 10^{-3}) were added to 5 ml. of broth (Bacto Penassay Broth, Difco) containing serial dilutions of drugs. The MIC values chosen were the lowest drug concentrations which inhibited visible growth after overnight incubation at 37° C.

Transfer of drug resistance. The nine chloramphenicol-resistant isolates were examined for the presence of R factors by the methods of Anderson & Lewis (1965*a*, *b*), using *Escherichia coli* K12F$^+$, K12F$^-$ and K12HfrH as recipient strains. Drug-resistant recipient colonies from these mating mixtures were isolated on plates containing the appropriate antibiotic, purified on MacConkey Agar (Difco), and their full resistance pattern was determined by a disk-diffusion method. The

fi (fertility inhibition) character was examined by the method described by Pitton & Anderson (1970), using phages $\mu 2$ and R17 (male-specific) and $\phi 2$ (female-specific). A second series of crosses was carried out, using resistant *E. coli* K12 as donor strains and *S. typhi* phage type A and *S. typhimurium* phage type 36 as recipient strains.

Mechanisms of resistance. The acetylating ability for chloramphenicol was determined by the method of Piffaretti (Piffaretti & Pitton, 1970).

Stability of the plasmid in its original strain of S. typhi. This was determined by the replica plating technique (Lederberg & Lederberg, 1952).

RESULTS

Antibiotic sensitivity tests

The nine isolates were resistant to chloramphenicol (C), streptomycin (S), sulfafurazole (Su) and tetracyclines (T) but sensitive to penicillins and cephalosporins as well as to kanamycin, gentamicin, neomycin and colomycin by the standard disk method. The MIC's of antibiotics for the wild-type strains were 120 μg./ml. for chloramphenicol, more than 200 μg./ml. for streptomycin, 100 μg./ml. for tetracycline, 3 μg./ml. for neomycin and gentamicin, and 6 μg./ml. for kanamycin. The MIC's for chloramphenicol were 160 μg./ml. in the K12 lines, 100 μg./ml. in *S. typhi* A, and 160 μg./ml. in *S. typhimurium*.

Transfer of drug resistance

The nine primary isolates transferred chloramphenicol resistance to *E. coli* K12 at a frequency of about 10^{-4}. All the K12 colonies selected on chloramphenicol showed the stable resistance pattern CSSuT. In the second series of crosses, all resistances were transferred at the same frequency, about 10^{-5}, into the two standard salmonellas, *S. typhi* Vi-type A and *S. typhimurium* type 36 (Anderson, 1966). This CSSuT resistance factor is *fi*$^-$ in K12HFrH lines when tested with the $\mu 2$ and R17 phages. In K12F$^+$, however, the R factor displaces the F factor, giving rise to F$^-$ cells. It thus shows one-sided incompatibility with F, since it is stable in, and seems not to affect the fertility of, Hfr strains, where the F factor is integrated into the chromosome. The same properties have been observed independently in this R factor by E. S. Anderson & H. R. Smith and by D. H. Smith (personal communications). This factor belongs to the 'H group' (Anderson & Smith, 1972; Grindley, Grindley & Anderson, 1972). It does not modify the sensitivity of K12F$^-$ lines towards the $\phi 2$ phage or produce phage restriction in *S. typhi* or *S. typhimurium*.

All the strains studied (the nine wild-type *S. typhi*, the K12F$^+$ and F$^-$ derivatives, and the *S. typhi* and *S. typhimurium* progeny obtained in the second crosses) acetylated chloramphenicol.

The CSSuT complex is probably not very stable in the wild-type strains, since the frequency of spontaneous loss of the resistance was about 3×10^{-3} for C and 10^{-3} for T.

DISCUSSION

All cultures of the chloramphenical-resistant strain of *S. typhi* isolated from this case have identical properties. They all react with the Vi-typing phages as degraded Vi-strains; and they carry the same *fi*$^-$ transferable R factor with a CSSuT resistance pattern. These results, as well as the clinical and epidemiological history of our patient, establish the identity of these isolates with those implicated in the outbreak of typhoid fever in Mexico (CDC Weekly Report, 1972*a*, *b*). Since Anderson & Smith (1972) have recently studied two C-resistant *S. typhi* cultures involved in this epidemic, it was of interest to determine whether our studies would show that the strain isolated in Switzerland was genetically indistinguishable from that found in Britain. No differences appeared: the Swiss strain belongs to the same Vi-type and carries the same R factor as those isolated in England.

These findings suggest that the Mexican epidemic of typhoid fever was caused by a single strain of *S. typhi* carrying the same R factor throughout. This has the resistance pattern CSSuT, belongs to group H, and displaces the F factor when the latter is in the extrachromosomal state.

An interesting finding in this case is the relatively high rate of spontaneous loss of C and T resistance in our *S. typhi*. This may explain the puzzling fact that one of the stool cultures performed after completion of the first course of antibiotic treatment yielded drug-sensitive *S. typhi*. This sensitive line had the same characteristics – phage type, biochemical and serological properties – as the resistant isolates.

We are indebted to Professor B. Courvoisier for allowing us to study this clinical case and to Dr Marie Sitavanc for isolation and microbiological identification of our strains. The expert technical assistance of Miss Janine Corlet is gratefully acknowledged. Our thanks are also due to Dr E. S. Anderson for his help in the genetic study of this R factor and in preparing this report.

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Interactions of TRIC agents with macrophages and BHK-21 cells observed by electron microscopy

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SUMMARY

TRIC agents do not multiply in mouse peritoneal macrophages in culture but have a toxic effect on them, whereas they multiply readily in BHK-21 cells. Sections of macrophages and of BHK-21 cells fixed during the first 90 min after inoculation were examined by electron microscopy. Macrophages ingested all forms of the organism, which were eventually degraded in lysosomes. However, elementary bodies were distinguished from other TRIC particles by the delay in their transfer to lysosomes. BHK-21 cells ingested elementary bodies selectively, and in these cells the organisms were neither found in lysosomes nor degraded. Instead they showed morphological changes that probably represented an early stage of development.

INTRODUCTION

The agents of trachoma and inclusion conjunctivitis (TRIC agents) multiply in various kinds of cell in culture but generally cause little or no cytopathic effect. However, if mouse peritoneal macrophages *in vitro* ingest even a small number of infective organisms per cell, the macrophages are killed (Taverne & Blyth, 1971). Such destruction of macrophages may contribute not only to the fatal effects of large doses of TRIC organisms given intravenously to mice but perhaps also to the cell damage that is seen in the conjunctiva in the natural disease, trachoma. It is likely that the toxic effect of TRIC organisms on macrophages is mediated through the lysosomal system, since it is accompanied by changes in the distribution of the lysosomal enzyme acid phosphatase(Taverne, Blyth & Ballard in preparation).

This report describes the events observed by electron microscopy in macrophages that have ingested TRIC organisms. The study was undertaken to determine the relationship of ingested organisms to the systems of cytoplasmic vacuoles of macrophages. A comparison is made with the early events observed after infection of BHK-21 cells, which support the multiplication of the organisms.

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METHODS

Cell culture

Methods for BHK-21 cells and for macrophages, together with infectivity titrations in BHK-21 cells, have been described (Taverne & Blyth, 1971).

Macrophages

These were obtained from the peritoneal cavity of mice of strain CS-1 without artificially stimulating the production of exudate.

TRIC agents

Although preliminary observations were made on cell cultures inoculated with material from infected yolk sacs, the results reported here were obtained from experiments with suspensions made from infected BHK-21 cells. Macrophage cultures were inoculated with strain HAR-2*f* (Taverne, Blyth & Reeve, 1964). For the inoculum BHK-21 monolayers infected 44 hr. earlier with a yolk-sac pool were disrupted, incubated with 0·5% trypsin for 30 min. at 37° C. and centrifuged at 8000 *g* for 20 min. The pellet was resuspended in phosphate buffered saline (pH 7·4) containing 0·25 M sucrose and 10 % calf serum and was stored at −70° C. The suspension contained $2{\cdot}4 \times 10^9$ inclusion-forming units/ml. For the experiment with BHK-21 cells, a suspension of strain MRC-4*f* (Taverne *et al.* 1964) was kindly supplied by Miss M. Harrison of the Trachoma Unit. It contained $6{\cdot}0 \times 10^8$ inclusion-forming units/ml. and $2{\cdot}0 \times 10^9$ total TRIC organisms/ml. (counted by the dark-ground technique of Reeve & Taverne, 1962).

Latex balls

These were obtained from Serva Entwicklungslabor, Heidelberg, and were 0·357 μm. in diameter.

Inoculation of cultures

Overnight cultures of BHK-21 cells or macrophages were inoculated with 1 ml. of TRIC suspension containing about 200 infective organisms per cell and centrifuged at 1000 *g* for 10 min. at 35° C. The medium was then changed and the cultures were returned to an atmosphere of 5% CO_2 at 35° C.

Electron microscopy

After removal of the medium, groups of 6 cultures were fixed for 30 min. at 0° C. with cacodylate buffer containing 0·3% OsO_4 and 1·7% glutaraldehyde (Hirsch & Fedorko, 1968). The cultures were rinsed with two changes of ice-cold 0·1 M sodium acetate at 5 min. intervals, and were then stained with 0·25% uranyl acetate for 30 min. at 0° C. After two further rinses the cells were scraped from the cover-slips and collected by filtration on 0·8 μm. membrane filters (Sartorius no. 11304). The cells were overlaid with a drop of agar solution and cooled on ice.

The filter disks carrying the cells were dehydrated in isopropanol which was replaced with xylene and then infiltrated with Araldite in which they were embedded. Thin sections were stained with lead citrate (Venable & Coggeshall, 1965).

Demonstration of acid phosphatase activity

The cultures were fixed in buffered glutaraldehyde, incubated in Gomori's lead nitrate-glycerophosphate medium and post-fixed with osmium tetroxide. After the cells had been scraped from the coverslips, dehydration and embedding were carried out as above.

RESULTS

Morphological composition of infective suspensions

Samples of suspensions used to inoculate cell cultures were deposited by centrifugation and sections of the deposits were examined in the electron microscope. In addition to TRIC organisms the suspensions contained cellular debris, which was more abundant in specimens from yolk-sac material than in those from BHK-21 cells. All the developmental forms of TRIC organisms, ranging from large reticulate bodies (RB) to small, easily recognized elementary bodies (EB) were present in both kinds of suspension.

Classification of TRIC particles

Preliminary experiments suggested that the intracellular fate of ingested particles depended, among other things, on their nature. This suggestion was investigated by localizing, identifying and counting the various types of particle within cells.

Although the population of TRIC organisms was made up of a continuous range of morphological forms, it was arbitrarily divided into three groups, namely EBs, RBs and condensed bodies (CBs) (Plate 1). This differentiation between the classes was necessarily subjective; it depended both on size (in relation to the estimated level of section through the particles) and on structure. Small intermediate forms that contained many ribosomes and no dark centre (nucleoid) were grouped with RBs, whereas those of a similar size whose contents showed definite condensation or few ribosomes were grouped with EBs. Condensed bodies were recognized to be a type of TRIC particle after it was found that there was a continuous series of forms from slightly distorted EBs that were a little denser than normal to severely shrunken and extremely dense particles (Plate 1*d*, *e*).

RBs and EBs were subdivided according to their condition; those considered to be imperfect are referred to as 'sick', for the sake of brevity. This separation was based on an assessment of such characters as shape, continuity of membranes and their relationship to the cytoplasm, structure of the cytoplasm and the amount of apparently empty space within the organism (Plate 1).

Entry into macrophages

In macrophages fixed immediately after the inoculation period many organisms were already enclosed within cytoplasmic vacuoles. Because inoculation involved centrifugation for 10 min., some of these organisms may have been inside the cell for most of this time. Other organisms were in the process of entering the cell, and many were found in a layer of inoculum deposited on the cell surface.

Particles entered the cell by phagocytosis. In many instances single organisms

were enfolded by small cytoplasmic processes so that they were transferred into the cell within a closely fitting membrane, accompanied by a minimum quantity of fluid (Plate 2*a*, *b*, *c*). All types of TRIC particle could enter singly and even a mitochondrion from the inoculum was found closely invested within a cytoplasmic vacuole, apparently after entering the cell in this way. In other instances larger cytoplasmic processes surrounded a portion of the deposited inoculum, enclosing a group of organisms of various types together with fluid and cell debris or yolk granules (Plate 2*d*). As a result, vacuoles containing a mixture of different types of material were carried into the cytoplasm.

Fate of organisms within macrophages

Two types of cytoplasmic vacuoles contained TRIC organisms. First, there were large irregularly shaped vacuoles that contained many particles of different sorts (Plate 3*a*). Although some of these vacuoles were the result of the phagocytosis of varied material in a single mass, some contained, in addition to TRIC particles, amorphous material identical with that found in vacuoles in uninoculated cells (Plate 3*c*). Its presence suggested that fusion had occurred between vacuoles containing TRIC particles and pre-existing lysosomes. That some of these vacuoles were lysosomes was confirmed by the Gomori procedure for demonstrating acid phosphatase activity (Plate 3*b*).

Other vacuoles contained one, or occasionally two, TRIC particles within a tightly investing membrane (Plate 4). The isolated particles were assumed to have entered singly. By analogy with the classification used for vacuoles enclosing ingested *Histoplasma capsulatum* (Dumont & Robert, 1970) we refer to the close-fitting vacuoles as 'tight' (T) vacuoles. Although there appeared to be a clear distinction between the two types of vacuole, a few were found that did not fit precisely into either category. These were round and larger than T vacuoles; they contained a single EB, or a single sick EB, surrounded by fluid and were most common in specimens fixed 40 or 80 min. after inoculation (Plate 3*d*). Since they contained only one organism they were classified as T vacuoles. As entry into BHK-21 cells appears to be entirely into T vacuoles (see below) special attention was directed to T vacuoles in macrophages and a quantitative survey was made of the fate of the TRIC particles within them. In the experiment selected for analysis, latex balls equal in number to infective organisms were added to the inoculum so that the reaction of the macrophage to TRIC particles and to a more inert particle could be compared. At various times after inoculation the particles were classified according to the system already described, and those within T vacuoles were distinguished from those in other vacuoles. For the sake of precision in classification the rare vacuoles containing more than one particle but otherwise identical with T vacuoles were not classified with them but were included with the other vacuoles.

All types of TRIC particles and latex balls were found within the cells at all times (Table 1). An indication of the reliability of the counts is given by the fact that throughout the period of observation the ratio of small TRIC particles (EBs, sick EBs and CBs taken together) to large particles (RBs and sick RBs) remained

Table 1. *TRIC particles counted in macrophages at intervals after inoculation**

Type of particle	TRIC pellet†	0 min.		20 min.		40 min.		80 min.	
		L	T	L	T	L	T	L	T
EB	28	16	10	37	14	18	15	3	4
CB	20	27	18	10	5	31	6	27	3
Sick EB	26	22	10	70	8	59	4	66	6
RB	67	0	10	5	1	1	2	1	0
Sick RB	14	52	7	51	3	66	2	56	0
Total TRIC	155	117	55	173	31	175	29	153	13
Latex balls	—	12	4	27	1	12	1	22	2

L: in vacuoles that resembled lysosomes. T: in 'tight' (T) vacuoles.

* Although approximately the same number of cell sections were examined at each time the volume of cytoplasm surveyed was not constant. Direct comparison between different times is therefore not valid.

† Numbers of particles in a representative inoculum (before addition of latex balls).

relatively constant. (No conversion of EBs into RBs should have occurred within 80 min. since this process requires at least 4 hr., even in a cell which supports the multiplication of TRIC organisms.) Immediately after inoculation about a third of all organisms were present in numerous T vacuoles; this proportion and the absolute number of T vacuoles decreased with time.

Direct comparison of the numbers of each class of TRIC particle in samples of macrophages taken at different times is not valid since, although about the same number of cell sections were examined in each case, their thickness inevitably varied. This difficulty cannot immediately be overcome by converting the numbers to proportions. For instance, suppose that the number of EBs in T vacuoles is expressed as the proportion of all EBs counted in that sample. The changes observed in this proportion with time would not simply reflect an altered relationship between EBs and T vacuoles, but could be affected by changes in the number of EBs resulting from, say, their conversion to sick EBs, if this occurred predominantly in one type of vacuole.

If all particles had an equal chance of being found in T vacuoles, then the number of particles of a particular class in T vacuoles, expressed as the proportion of all particles found in these vacuoles (the 'observed' proportion) would approximate to the total number in that class expressed as a proportion of all intracellular particles, the latter being an estimate of the 'expected' proportion. The ratio of the observed proportion of each class found in T vacuoles to its expected proportion gives a measure by which the behaviour of the different classes can be compared. This ratio allows valid comparisons to be made both within one sample and between different samples. A ratio greater than 1 indicates that more particles of that class were found in T vacuoles than would be expected if the relationship of all types of particles to T vacuoles was the same.

The proportions of various types of particle in T vacuoles at various times are compared with the proportions in both types of vacuole taken together in Fig. 1. Each pair of columns represents the counts of intracellular particles at a particular time after inoculation. The left-hand column represents the proportions of each

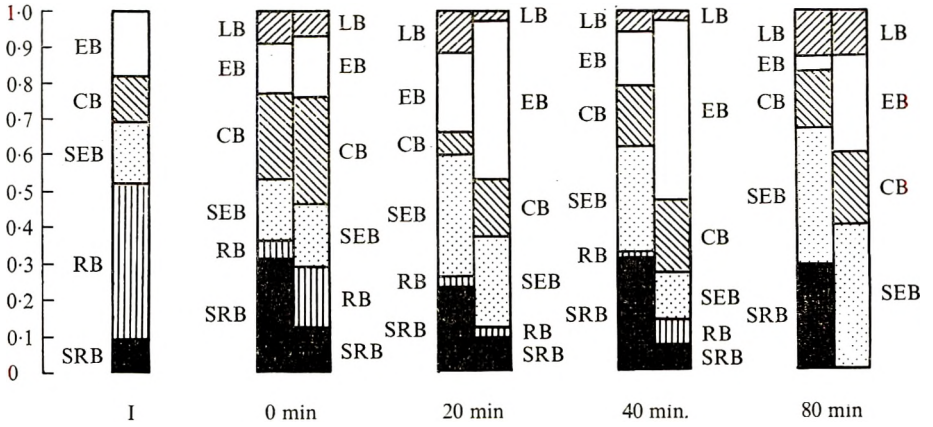

Fig. 1. The proportions of different types of TRIC particles in macrophages at intervals after inoculation. The single column marked I shows the proportions in a representative inoculum before the addition of latex balls. The left-hand column of each pair represents the proportions in all vacuoles at each time; the right-hand column shows those in 'tight' (T) vacuoles. The proportions are derived from the numbers in Table 1. EB, Elementary body; CB, condensed body; SEB, sick elementary body; RB, reticulate body; SRB, sick reticulate body; LB, latex balls.

Table 2. *The ratio of the observed number of particles in T vacuoles to the expected number, assuming random distribution between all vacuoles*

	0 min.	20 min.	40 min.	80 min.
EB	1·15	1·41*	1·68*	1·88*
CB	1·19	1·51	1·09	1·13
Sick EB	0·99	0·83	0·59	1·03
RB	∞ *	1·12	1·86	0·00
Sick RB	0·46*	0·54	0·32*	0·00*
Latex balls	0·84	0·37	0·67	1·00

* These ratios differ significantly from 1·00 ($P<0·05$) using the χ^2 test with Yates's correction.

type of particle in all vacuoles and the single column on the extreme left shows the morphological composition of a representative suspension of organisms without latex balls. Thus the composition of the inoculum can be compared with that of the intracellular particles at different times after inoculation. The right-hand column of each pair shows the proportions in T vacuoles only. If all classes of particle had the same chance of being in T vacuoles the proportions shown in the left-hand column should provide an estimate of those to be expected in the right-hand column, and the columns should match.

The ratio of the height of a segment in the right-hand column to the height of the corresponding segment in its left-hand neighbour is the measure of behaviour referred to above. To aid comparison between different classes of particles at different times this ratio is tabulated (Table 2). When the numbers of intracellular particles in each class and the proportion in T vacuoles were analysed the following points emerged:

There were always more normal EBs in T vacuoles than expected and this

Table 3. *TRIC particles counted in BHK-21 cells immediately after inoculation and 80 min. later**

Type of particle	TRIC pellet†	0 min.	80 min.
EB	30	36	51
CB	19	31	13
Sick EB	16	5	7
RB	28	1	3
Sick RB	40	0	1
Total TRIC	133	73	75
Latex balls	—	0	0

* All particles were in T vacuoles. Direct comparison between counts at different times is not valid (see note to Table 1).

† The numbers of particles in the inoculum (before the addition of latex balls).

discrepancy increased with time. Condensed bodies were the only other particles that were always found in T vacuoles more frequently than expected. Immediately after inoculation the number of EBs and of sick EBs expressed as a proportion of the total particles found in the cells compared well with that in the inoculum, but by 80 min. the proportion of normal EBs was noticeably decreased, whereas that of sick EBs was increased (Fig. 1). The ratio of EBs to sick EBs decreased continuously with time. Thus normal EBs were apparently being replaced by 'sick' ones.

By far the greater proportion of RBs in the cells were classified as 'sick' and were found in lysosomes. Normal RBs were seldom found intracellularly except immediately after inoculation when they were all in T vacuoles.

Somewhat fewer latex balls than normal EBs were seen within cells, although the number added had been calculated to equal the number of infective EBs. However, it is probable that some EBs classified as 'normal' were not infective, so that this result does not imply that EBs and latex balls were ingested with differing efficiency. The great majority of the latex balls were in lysosomes.

Entry into BHK-21 cells

In BHK-21 cells fixed immediately after the inoculation period small TRIC particles, both EBs and CBs, were readily found either in the process of entering the cell or within cytoplasmic vacuoles. Although reticulate bodies were abundant in the inoculum (Plate 1*a*) and were observed outside infected cells, few were found in cytoplasmic vacuoles (Table 3; Fig. 2) and most of these contained an early nucleoid, indicating that they were probably forms intermediate between EBs and RBs. Debris from the inoculum was seldom phagocytosed by BHK-21 cells, and although the inoculum contained latex particles equal in number to infective particles, none were found intracellularly.

The surface of BHK-21 cells was smooth in comparison with that of macrophages and all particles apparently entered singly; phagocytosis of groups of particles by means of large cell processes was not observed.

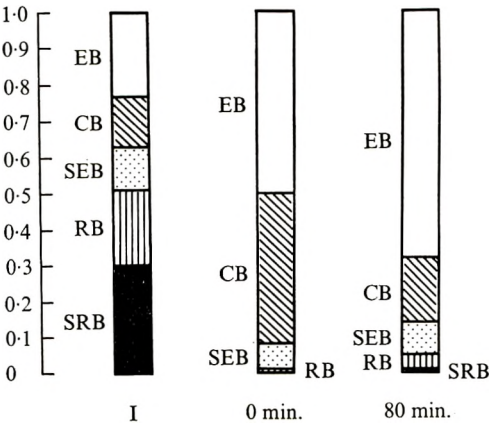

Fig. 2. The proportions of different types of TRIC particles in BHK-21 cells at intervals after inoculation. The column marked I shows the proportions in the inoculum before the addition of latex balls. The remaining columns represent the proportions in the cells, at each time; all the particles were in 'tight' (T) vacuoles. No latex balls were found within BHK-21 cells in this experiment. The proportions are derived from the numbers in Table 3. For abbreviations see Fig. 1.

Fate of organisms within BHK-21 cells

At the two times when samples were taken, TRIC particles or their residues were not found in lysosomes. However, lysosome-like vacuoles were not common in our BHK-21 cells, whether or not the cells were infected. Cytoplasmic vacuoles containing TRIC particles were of only one type; they contained one (or in rare instances, two) TRIC particles within a tightly investing membrane (T vacuoles) (Plate 4*a*, *b*). Sometimes the vacuoles also contained a few small irregular vesicles. Immediately after inoculation the vacuoles were situated at the periphery of the cell, but 80 min. later groups were found concentrated to one side of the nucleus. They lay in or near a zone relatively free from organelles and containing intracellular fibrils and polyribosomes, adjacent to the Golgi apparatus. Nearly all the EBs were classified as healthy, but in many of them the cell wall had separated from the cytoplasmic membrane leaving an electron-transparent zone surrounding the cytoplasm, which itself appeared normal (Plate 4*b*). Thus the overall diameter of the organism increased while that of its cytoplasm remained unaltered. Quantitative analysis demonstrated that immediately after inoculation a higher proportion of the intracellular particles were CBs than 80 min. later (Table 3; Fig. 2). At the earlier time a few vacuoles containing EBs or CBs were surrounded by small vesicles about 50 nm. in diameter, some of which were fusing with (or budding from) the vacuolar membrane. These vesicles resembled the small Golgi vesicles implicated in the synthesis and concentration of secretion products. After 80 min. more of the vacuoles that contained TRIC particles were associated with small vesicles, and more vesicles surrounded each vacuole (Plate 4*b*).

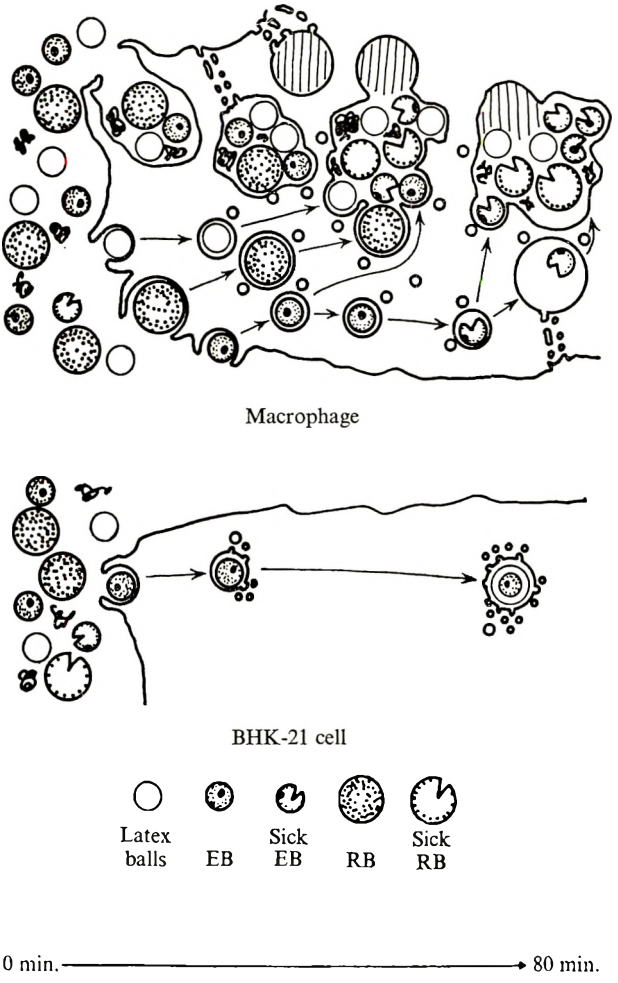

Fig. 3. Diagram of the sequence of events in macrophages and BHK-21 cells following inoculation with TRIC and other particles.

DISCUSSION

A diagram showing our interpretation of the sequence of events during and after entry of TRIC organisms into macrophages and BHK-21 cells is presented (Fig. 3); the main features which distinguish the reactions of macrophages to these organisms from the reactions of BHK-21 cells are listed (Table 4). One of the most striking contrasts is that macrophages rapidly ingested all kinds of particles whereas BHK-21 cells ingested EBs and CBs selectively to a remarkable degree. Conditions of culture can of course materially alter the efficiency with which cells ingest particles, but it is unlikely that the different experimental conditions used for handling macrophages and BHK-21 cells would account for the differences in phagocytic ability observed here.

Both kinds of cell take in particles singly. For macrophages this is the commonest form of entry, and their lower selectivity results primarily from their ability to

Table 4. *Differences between macrophages and BHK-21 cells in their interactions with TRIC organisms*

Macrophages	BHK-21 cells
All types of particle enter by phagocytosis	EBs (and CBs) enter by selective phagocytosis
Organisms and other particles enter singly and in groups	Organisms enter singly
TRIC organisms found singly in close-fitting vacuoles, or with other material in phagolysosomes	TRIC organisms found singly in close-fitting vacuoles only
EBs degraded in lysosomes	EBs neither transferred to lysosomes nor degraded
Small vesicles do not accumulate around close-fitting vacuoles containing single TRIC organisms	Small vesicles accumulate around and fuse with, vacuoles containing TRIC organisms
Organism does not show early signs of development	Wall of TRIC organism separates from its plasma membrane (early development?)
Organisms do not multiply	Organisms multiply
Organisms are toxic to the cells	Organisms are not toxic to the cells

ingest a wider range of particles in this way. In addition macrophages, unlike BHK-21 cells, ingest particles in groups, a type of entry that is obviously not selective. BHK-21 cells ingested latex balls much less efficiently than EBs and CBs (although 131 EBs and CBs were counted in the cells, no latex balls were found) whereas macrophages ingested EBs and latex balls equally well. However, latex balls can enter BHK-21 cells in some circumstances (unpublished experiments). It is clear that both EBs and CBs have a specific stimulating effect on phagocytosis which, at least in BHK-21 cells, is superior to that possessed by either RBs or latex balls equal in size to EBs. Since this property cannot be due solely to the size of the particles, it must reside in some physicochemical property of their external surface.

That both EBs and CBs are ingested by BHK-21 cells in preference to other types of particle and that once inside the cell both are treated in the same way – both by macrophages and by BHK-21 cells – supports our recognition on morphological grounds that they are closely related. It is possible that CBs are damaged EBs that are not viable, but it seems more likely that they represent a proportion of EBs that react differently to fixation so that osmotic changes cause more distortion and shrinkage. If, in addition, EBs become more permeable to solutes used in preparation for electron microscopy during the early stages of development so that fewer shrink, then the observation that the ratio of CBs to EBs in BHK-21 cells decreases with time (Table 3) supports the hypothesis that some CBs are viable EBs. A similar phenomenon can be seen during the developmental cycle of *Rickettsiella melolonthae* where some of the mature organisms, when observed by electron microscopy, appeared as denser and smaller forms than the multiplying

organism (Devauchelle, Meynadier & Vago, 1972). Again, when Gram-negative bacteria are prepared for electron microscopy, some organisms appear dense with convoluted membranes while others from the same culture appear larger with a smooth profile.

Once within the cells, the main difference between the response of macrophages and that of BHK-21 cells to TRIC organisms is that EBs and CBs are transferred into lysosomes in the former but not in the latter. This is demonstrated by the presence within macrophages of degraded EBs in vacuoles, some of which were shown to contain a lysosomal enzyme. In these cells, the ratio of EBs to sick EBs decreases with time but in BHK-21 cells it remains constant over the same time interval and no degraded EBs are found in lysosome-like vacuoles.

It is usually assumed that the fate of a particle after phagocytosis is to enter a digestive vacuole, but there are exceptions to this rule. Intracellular parasites must escape digestion within their host cell either because they are resistant to the action of lysosomal enzymes or because they avoid contact with them. Both these mechanisms of resistance exist among the *Mycobacteria*. For instance, *M. lepraemurium* is clearly resistant to the action of the enzymes since it multiplies within lysosomes (Brown & Draper, 1970); indeed its multiplication in rat fibroblasts was enhanced when the conditions of culture increased lysosomal activity (Brown, Draper & D'Arcy Hart, 1969). By contrast, living organisms of *M. tuberculosis* strain H37Rv multiplied in macrophages within vacuoles that did not fuse with lysosomes, although damaged organisms entered lysosomes and were digested (Armstrong & D'Arcy Hart, 1971). Rickettsiae avoid contact with lysosomal enzymes in one of two ways: either, like *Rickettsia sennetsu* and *Rickettsiella melolonthae*, they multiply in vacuoles that remain separate from lysosomes (Anderson, Hopps, Barile & Berheim, 1965; Devauchelle *et al.* 1972), or, like *Rickettsia prowazeki* and *Rickettsia rickettsii*, they escape into the cytoplasm of the host cell (Anderson *et al.* 1965). In macrophages parasitized by the protozoon *Toxoplasma gondii*, two populations of organisms were seen: about half the organisms degenerated in typical phagocytic vacuoles containing acid phosphatase, whereas the rest were enclosed in vacuoles that contained no acid phosphatase, remained morphologically normal and eventually divided (Jones & Hirsch, 1972).

TRIC organisms escape digestion in BHK-21 cells because the vacuole in which they multiply is not a lysosome. Other Chlamydia also appear to prevent the transfer of lysosomal materials to phagocytic vacuoles as was shown by Friis (1972) for the meningopneumonitis agent in L cells. Either the TRIC agent actively inhibits fusion of its vacuole with lysosomes or it lacks a factor that stimulates fusion. This failure of fusion in BHK-21 cells is unlikely to result solely from the lower concentration of lysosomes in the host cell cytoplasm (thus decreasing the probability of collision) because vacuoles containing TRIC particles accumulate near the nucleus where they are close to lysosomes and to the Golgi apparatus, which is the source of vesicles containing the acid hydrolases for the lysosomal system.

Close examination of our results reveals that, in macrophages, EBs persist longer in T vacuoles than other types of particle. For instance, RBs were trans-

ferred to lysosomes and degraded so rapidly that normal RBs were rarely found intracellularly. Thus, as in BHK-21 cells, EBs are treated differently from other particles, since their entry into lysosomes is delayed; even so the majority of EBs are transferred to lysosomes and degraded within 80 min. of entry.

The absence of multiplication of TRIC organisms in macrophages may be associated with their destruction in lysosomes, but why they enter lysosomes in macrophages but not in BHK-21 cells is obscure. The simplest hypothesis, that vacuoles containing the organism escape fusion in BHK-21 cells because lysosomes are rare, is unlikely for the reasons given above. Another possibility is that the organism begins to develop in T vacuoles within macrophages as it does in BHK-21 cells but that in macrophages the development is abortive and as a result the vacuoles fuse with lysosomes. The enlarged T vacuoles containing a single EB may result from fusion of pinocytotic vesicles that contain ingested culture medium with the T vacuole, and would thus represent the first stage of the recruitment of the vacuole into the lysosomal system. Two changes were observed in infected BHK-21 cells 80 min. after inoculation that were never seen in macrophages at any time; one concerned the morphology of EBs, the other the interaction of the host cell cytoplasm with T vacuoles containing EBs. The separation of the cell wall from the cell membrane of developing EBs was seen only rarely at the end of the inoculation period, affected most particles by 80 min., and was no longer evident 6 hr. after inoculation (unpublished observations). The electron-transparent zone seems likely to result from the expansion of the cell wall of the organism and its appearance may be associated with the alteration in permeability of EBs that occurs (at least in the case of the meningopneumonitis agent growing in L cells) within one hour after the end of the infection period (Tamura, 1971).

The small vesicles clustered around T vacuoles containing EBs also appear to be associated with the development of the organism; they do not contain acid phosphatase (unpublished observations). In macrophages T vacuoles were never surrounded by these small vesicles although larger vesicles were common throughout the cytoplasm.

The morphological events reported here suggest that entry of EBs into lysosomes is the key to their toxic effect on macrophages, which probably results from a reaction between infective EBs and the lysosomes in which they lie. Again, entry into lysosomes is likely to be the essential step required for inactivation of the organism. By contrast, EBs never enter lysosomes in BHK-21 cells so that no toxic effect occurs and the organism remains free to multiply.

We would like to thank Mrs Anne Martin, Mrs Anne Fitzpatrick and Mr Ronald Ballard for their excellent technical assistance.

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EXPLANATION OF PLATES

Trachoma particles and vacuoles are classified according to the scheme described in the text. EB, Elementary body; CB, condensed body; RB, reticulate body; T vacuole, 'tight' vacuole.

PLATE 1

(*a*) A representative micrograph of a pellet of an inoculum prepared from BHK-21 cells infected with TRIC organisms. Arrows indicate forms intermediate between RBs and EBs.

(*b*)–(*g*) Individual particles from an inoculum, all at the same magnification. (*b*) EB, (*c*) sick EB, (*d*) and (*e*) CB, (*f*) RB, (*g*) sick RB.

PLATE 2

Ingestion of particles by macrophages.

(*a*) TRIC particles are apparently adsorbed to the cell membrane, which in one instance is indented.

(*b*), (*c*) Two stages in the phagocytosis of single particles.

(*d*) A cytoplasmic process engulfs a mixed group of particles.

PLATE 3

Particles within lysosomes in macrophages.

(*a*) Different types of particle are within a single lysosome with membranous debris which may have originated from TRIC organisms.

(*b*) This lysosome is in a cell treated by the Gomori technique. It contains damaged TRIC particles (arrows) together with a specific precipitate indicating localized acid phosphatase activity.

(*c*) This lysosome contains two CBs and a damaged RB as well as amorphous material which is probably condensed serum protein from the culture medium.

(*d*) This EB lies in an enlarged T vacuole. Several coated vesicles (possibly primary lysosomes; Novikoff, Novikoff, Quintana & Hauw, 1971) lie near the vacuole, but this association was rare and not restricted to this type of vacuole.

Plate 4

Particles within T vacuoles in BHK-21 cells and macrophages, all at the same magnification.
(*a*) An EB immediately after entry into a BHK-21 cell.

(*b*) Three organisms in BHK-21 cells 80 min. after inoculation. The EB on the right shows separation between its wall and its cell membrane. The two bodies marked with arrows are assumed to be tangentially sectioned T vacuoles containing TRIC particles. All three vacuoles are surrounded by small cytoplasmic vesicles.

(*c*)–(*g*) Particles in macrophages.

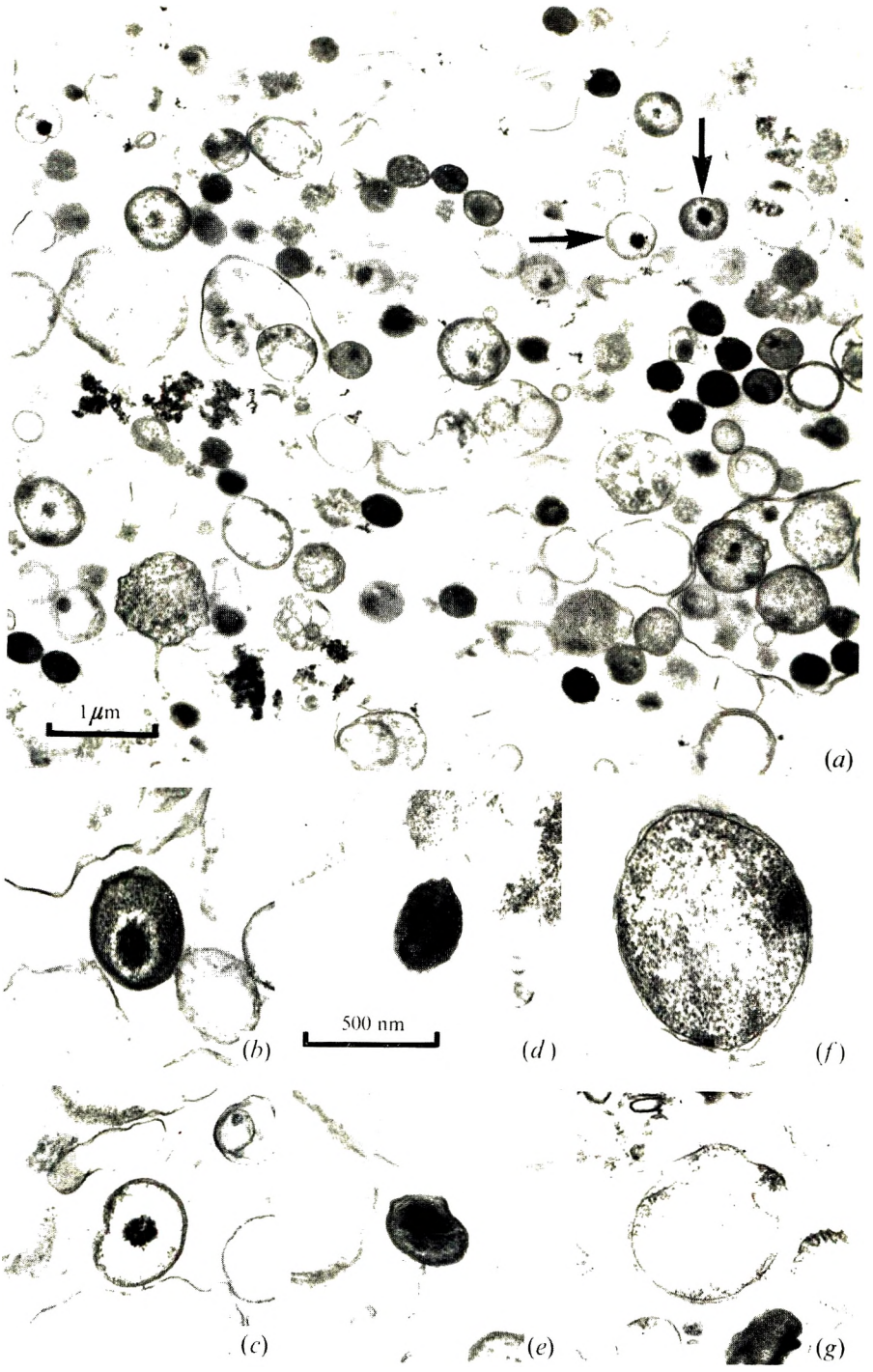
(*c*) An EB 20 min. after inoculation.

(*d*) A sick EB lying in an enlarged T vacuole 80 min. after inoculation.

(*e*) An RB immediately after inoculation.

(*f*) A latex ball immediately after inoculation.

(*g*) A CB 20 min. after inoculation.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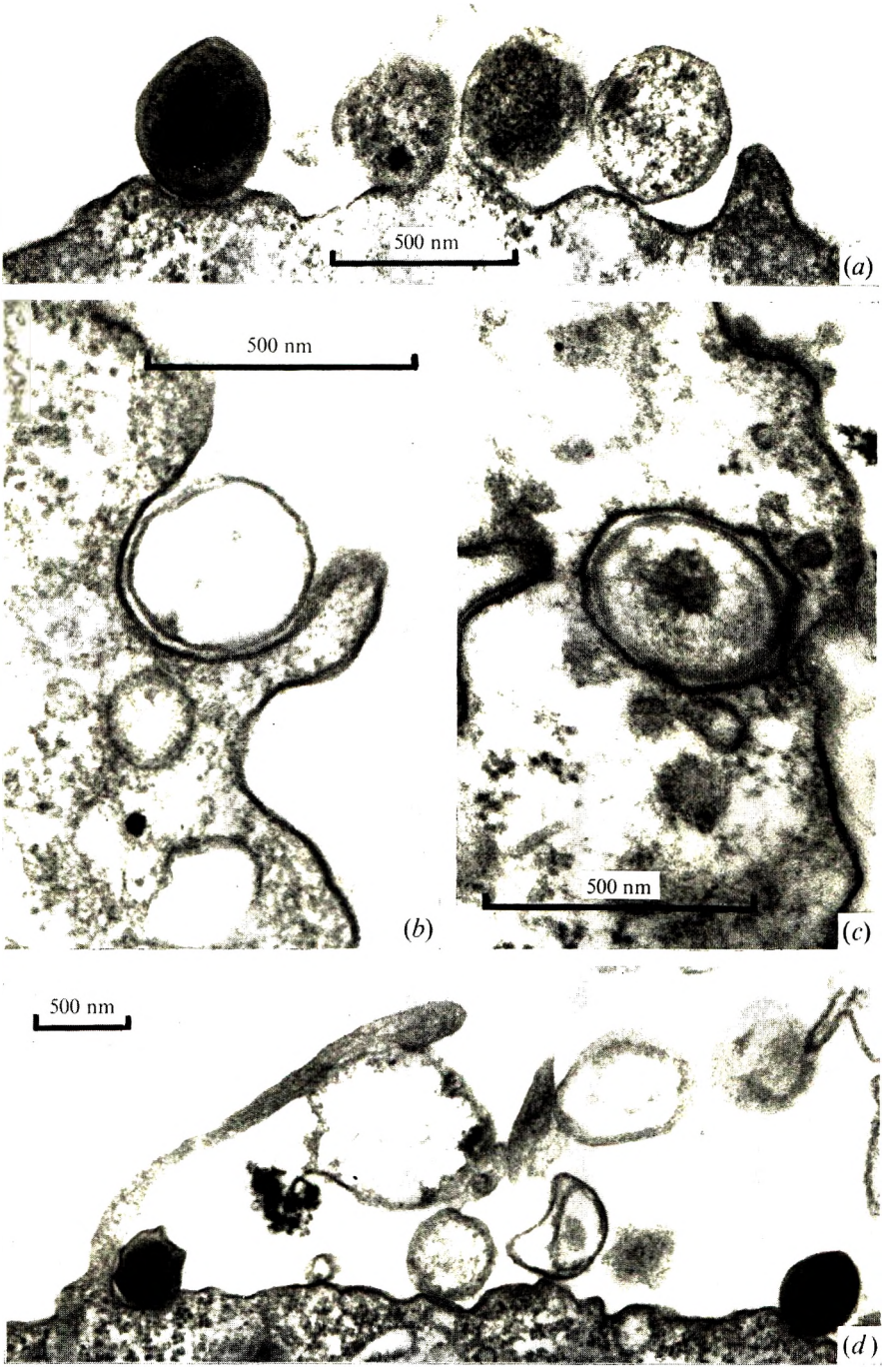
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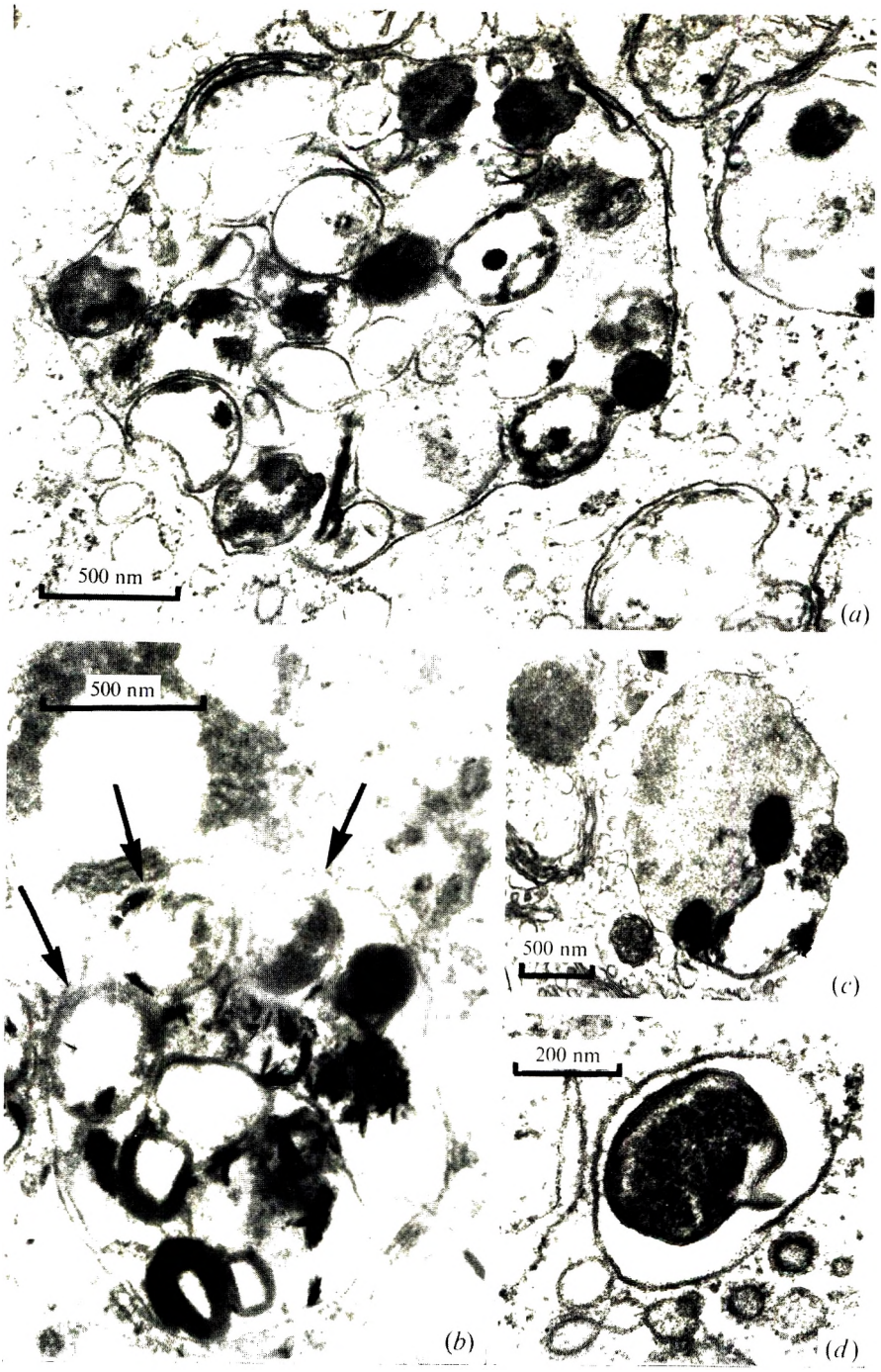
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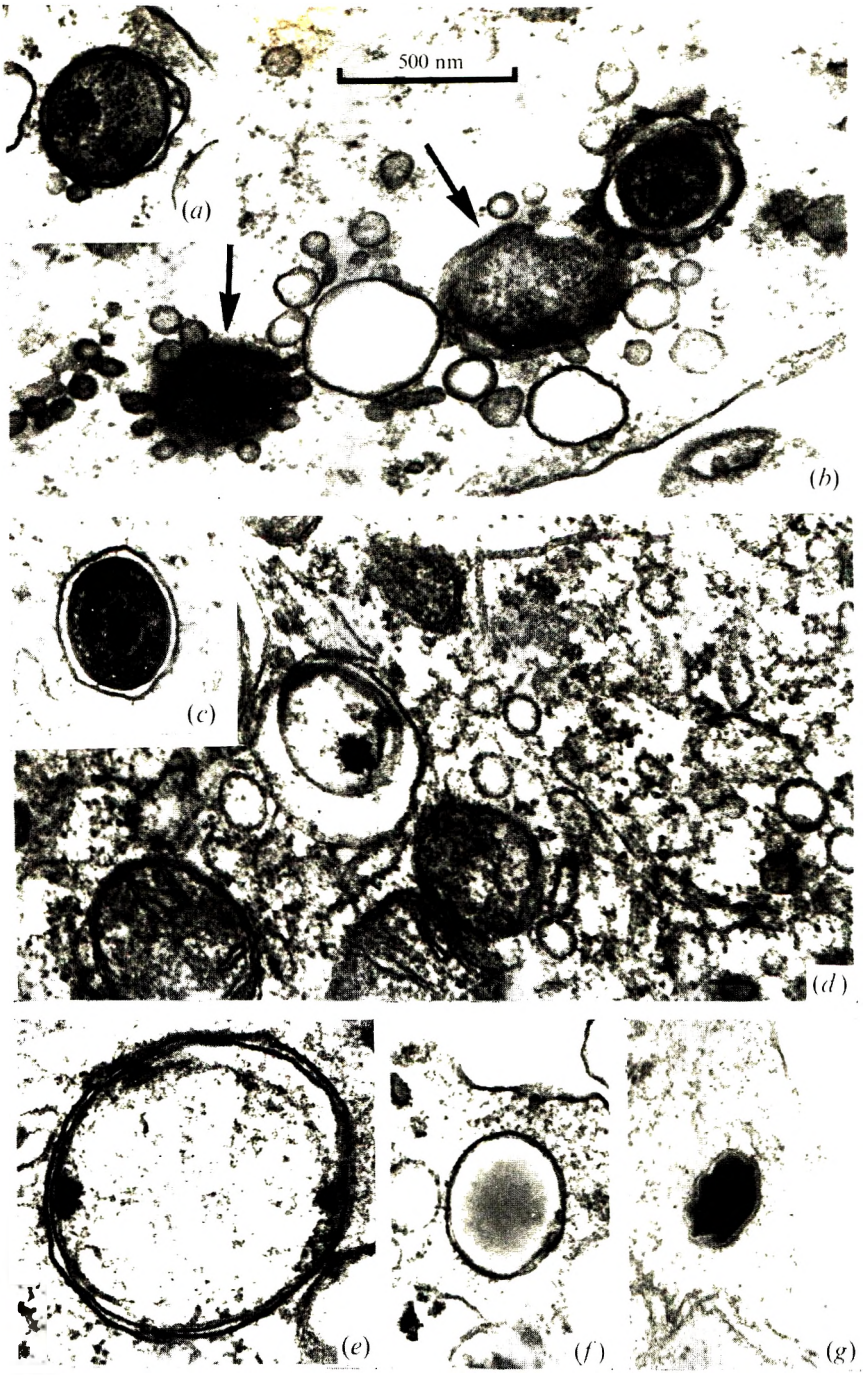
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Effect of stay in hospital and oral chemotherapy on the antibiotic sensitivity of bowel coliforms

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SUMMARY

The effects of oral chemotherapy and stay in hospital on the antibiotic resistance patterns of faecal coliform flora were studied. The coliform flora of 64% of 25 patients who were not receiving antibiotics was sensitive to all drugs tested. Hospitalization alone did not affect this proportion. The administration of tetracycline or ampicillin to patients, whether at home or in hospital, significantly increased the percentage of resistant bowel coliforms. Tetracycline showed a significantly greater effect than ampicillin. There was no significant increase in the percentage of patients with resistant flora after treatment with amoxycillin.

INTRODUCTION

The aerobic Gram-negative normal faecal flora is composed of bacterial genera which are all capable of acquiring plasmids carrying genes coding for drug resistance (R factors). This flora therefore is a huge potential reservoir of resistant organisms which may cause infection in other sites. Factors which affect the antibiotic sensitivity of these faecal organisms will therefore be of importance in determining the resistance patterns of subsequent infections caused by Enterobacteriaceae.

Studies on the flora of normal people not in hospital have shown that bacteria carrying R factors (i.e. R+) are present in a variable proportion of individuals (Datta, 1969; Datta *et al.* 1971; Moorhouse, 1969). In domiciliary patients it was shown that after the oral administration of tetracycline there was a significant increase in the antibiotic resistance of the faecal flora, but this effect was not nearly so great after ampicillin or sulphonamide therapy (Datta *et al.* 1971).

This study describes firstly the incidence of antibiotic-resistant bacteria in the bowel of domiciliary patients in the region of Edinburgh, some of whom had received anti-microbial therapy and others who had not. Secondly, it examines the effect on the bowel flora of the oral administration of anti-microbial agents to hospital patients, and thirdly, the effect of stay in hospital alone on the faecal flora.

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METHODS

The survey

Patients studied were those chosen to investigate the use of amoxycillin in pneumonia and chronic bronchitis (Jones *et al.* 1973). They were admitted to the City Hospital, Edinburgh, in 1971. On admission a rectal swab was taken from each patient and a note made of any anti-microbial treatment which the patient was currently being given. These patients were divided into groups according to which drug had been given before admission to hospital. There were 31 patients who had received ampicillin, 27 who had received tetracycline for less than 1 month, 26 who had received tetracycline for more than 1 month and 64 patients who had received no chemotherapy. The specimen taken at the time of admission was considered to reflect the patient's faecal flora outside hospital.

The 64 patients who had received no antibiotics were studied further. They were allocated to one of three treatment groups (see Jones *et al.* 1973): 23 patients were given tetracycline, 21 ampicillin, and 20 amoxycillin, a new semi-synthetic penicillin related to ampicillin (Sutherland, Croydon & Rolinson, 1972).

Another group of 25 patients, admitted to the same wards for conditions other than pneumonia or chronic bronchitis, and who did not receive antibiotics before or after admission, served as controls. The faecal flora of this control group on admission to hospital was compared with that of the 84 patients who had had antibiotics outside hospital and on discharge with that of the 64 patients who had had antibiotics in hospital.

From the latter two groups a portion of the last faecal specimen passed in hospital was also collected.

Bacteriology

Faecal specimens were spread on MacConkey agar plates for single colonies. Ten separate lactose-fermenting colonies were picked, and identified by ability to produce indole, split urea or use citrate as the sole carbon source. These organisms as a group will be referred to in the remainder of this paper as coliforms. The antibiotic sensitivity of each organism was determined. Oxoid DST agar (CM 261) plates containing 4% lysed blood and one of the following drugs – ampicillin 25 μg./ml., streptomycin 15 μg./ml., tetracycline 10 μg./ml., chloramphenicol 25 μg./ml., kanamycin 10 μg./ml., sulphadimidine 100 μg./ml., nalidixic acid 25 μg./ml., trimethoprim 2 μg./ml. and gentamicin 8 μg./ml. – were inoculated with approximately ten organisms of each strain, using a multiple inoculator, and incubated overnight. A known sensitive and resistant organism were included on each plate. The predominant faecal flora was considered sensitive if all ten colonies picked were sensitive to all the drugs tested. The flora was considered predominantly resistant if five or more of the colonies picked were resistant to at least one of the antibiotics tested. An organism resistant to at least three of the drugs tested was recorded as multi-resistant.

Table 1. *Effect of oral chemotherapy on bowel flora of patients living in the general community*

Antibiotic therapy	No. of patients (10 strains from each patient)	No. of patients with: Sensitive flora (0/10 strains resistant)	No. of patients with: Majority of flora resistant (at least 5/10 strains resistant to at least 1 drug)
None	25	16 (64)	6 (24)
Ampicillin	31	9 (29)	15 (48)
Tetracycline for less than 1 month	27	8 (29)	16 (59)
Tetracycline for more than 1 month	26	2 (8)	22 (84)

Figures in parentheses is percentage of patients.

Table 2. *Antibiograms of multi-resistant organisms obtained from patients in the general community*

Resistance pattern	No. of strains antibiotic therapy: Long-term tetracycline	Short-term tetracycline	Ampicillin
AST	20	—	—
ATC	10	—	—
ASSu	2	—	10
ATSu	1	2	—
STSu	8	10	1
TCSu	—	2	—
ASTSu	11	—	27
ATKSu	—	2	—
STCSu	—	18	—
STKSu	—	2	—
ATCSu	—	1	—
ATCKSu	—	1	—
ASTKSu	—	5	—
ASTCSu	1	19	7
ASTCKSu	1	12	—
Total	54	74	45

A, Ampicillin; S, streptomycin; T, tetracycline; C, chloramphenicol; K, kanamycin; Su, sulphonamide.

RESULTS

Table 1 shows the effect of administration of antibiotics on the faecal flora of 109 patients living in the general community. Of the 25 patients studied who were not receiving antibiotics, 64% had a sensitive flora (i.e. all 10 strains examined were sensitive to all drugs tested), 6 showed a predominantly resistant flora (at least 5/10 strains resistant) and no multi-resistant strains were isolated. The flora of patients who were on antibiotics before admission showed significant differences from those not receiving antibiotics. Only 29% of ampicillin-treated patients had a sensitive flora (difference from untreated group is significant, $P < 0{\cdot}025$). Of 27

Table 3. *Effect of hospitalization and oral chemotherapy on bowel flora of 89 patients admitted to City Hospital, Edinburgh*

Antibiotic given in hospital	No. of patients (10 strains from each patient)		No. of patients with: Sensitive flora (0/10 strains resistant)	No. of patients with: Majority of flora resistant (at least 5/10 strains resistant to at least 1 drug)
—	25	On admission	16 (64)	6 (24)
		On discharge	16 (64)	6 (24)
Tetracycline	23	On admission	9 (39)	10 (43)
		On discharge	1 (4)	22 (96)
Ampicillin	21	On admission	11 (52)	6 (29)
		On discharge	6 (29)	8 (38)
Amoxycillin	20	On admission	8 (40)	7 (35)
		On discharge	6 (30)	10 (50)

Figures in parentheses are percentage of number of patients.

patients treated with tetracycline for less than 4 weeks, 8 had sensitive flora ($P < 0{\cdot}05$) and of the 26 patients who had received tetracycline for a longer period, only 2 had sensitive flora ($P < 0{\cdot}001$). There was no statistical difference in the effect of ampicillin compared with tetracycline on the proportion of patients with sensitive flora (Table 1, column 3). However, tetracycline, whether given for a long or short time, significantly increased the likelihood of the majority of the flora being resistant compared with the untreated groups ($P < 0{\cdot}025$ if treated for less than one month and $P < 0{\cdot}001$ if long-term therapy used) (Table 1, column 4). Ampicillin did not show this effect.

There was a variety of resistance patterns amongst those strains resistant to three or more antibiotics and the patterns are shown in Table 2. There were no organisms found resistant to gentamicin, trimethoprim or nalidixic acid. There was no evidence of a particular antibiotic selecting a particular pattern. However, considering organisms resistant to only one or two antibiotics, there was a much higher incidence of excretion of tetracycline-resistant organisms in those patients receiving tetracycline than of ampicillin resistance in those receiving ampicillin.

Table 3 shows the effect of antibiotics on patients admitted to the City Hospital who had previously not received antibiotic therapy. There were 89 patients studied. Using the chi-square test there is no statistical significance between these groups of patients with respect to having sensitive flora on admission. Of the group who received no chemotherapy, there was no change at the time of discharge in the number of patients with sensitive flora. However, of patients treated with tetracycline there is a highly significant ($P < 0{\cdot}001$) decrease in the number with a sensitive flora and an increase in the number with the majority of the faecal flora antibiotic resistant ($P < 0{\cdot}001$). Ampicillin alters the faecal flora in the same way only to a lesser extent than tetracycline ($P < 0{\cdot}01$). In this series there was no

Table 4. *Duration of stay in hospital*

	Chemotherapy			
	None	Amoxycillin	Tetracycline	Ampicillin
No. of patients	25	20	23	21
Average stay (days)	14·2	16·5	12·3	12·1
Median stay (days)	13	15	13	12

statistically significant decrease in the number of patients whose faecal flora was still sensitive after chemotherapy with amoxycillin. The resistance patterns of the resistant organisms were similar to those occurring in patients prior to admission to hospital. There were no organisms isolated which were resistant to gentamicin, trimethoprim or nalidixic acid.

DISCUSSION

The carriage of R+ coliforms in faecal flora of normal people has been well established but the percentage of resistant organisms has varied enormously in different studies. Datta (1969) found that 52% of 100 patients from the London area who had not received chemotherapy within the previous 6 months, nor been in hospital, carried R+ bacteria, whereas Moorhouse (1969) found the much higher incidence of 81% among Irish infants (but some of these had had previous chemotherapy). In this study we found that in the Edinburgh region 36% of people not receiving antibiotics carried resistant coliforms in the faecal flora and in only one-quarter were they the predominant flora. Multi-resistance was very rare in people not receiving chemotherapy. Admission to hospital alone did not significantly alter the proportion of patients with resistant flora nor the resistance patterns of the coliform population of the bowel. (The average duration of stay in hospital was similar for all groups: see Table 4.) However, chemotherapy significantly changed the bowel flora whether the patient was in hospital or not. Tetracycline caused the greatest change. Ampicillin produced a similar but smaller change but with amoxycillin no change was demonstrated.

The origin of these resistant strains is of interest. It is possible that they are part of the minority flora before therapy and that appropriate drugs inhibit the sensitive flora allowing over-growth of minority strains. This point needs further elucidation.

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Hong Kong influenza in the Royal Air Force 1968–70

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SUMMARY

A prospective serological and clinical study of the epidemics due to the A2/Hong Kong/68 influenza virus was made during the winters 1968–9 and 1969–70 in volunteer subjects in the Royal Air Force. In October 1968 nearly all subjects had haemagglutination inhibiting (HI) antibody to the A2/Singapore/57 virus and more than half had antibody to strains more recently prevalent in Britain. The proportion with HI antibody to A2/Hong Kong/68 increased from 31 % in October 1968 (most at low titres) to 44 % after the first epidemic and 72 % after the second (most at high titres). Serological infection rates were much lower in those who had detectable antibody at the beginning of each winter than in those who did not. Respiratory illnesses coupled with serological evidence of influenza infection during the winter were rare in persons with an initial titre of HI antibody of 1/40 or more. Infection in the first winter conferred complete protection against infection, with or without illness, in the second. In both epidemics about half those with serological evidence of infection had no reported illness.

INTRODUCTION

Epidemics caused by the influenza A2 virus have occurred in Britain during most winters since the subtype first appeared in 1957. Meanwhile, the antigenic composition of the virus has gradually drifted from the original, with alterations being detected in relatively small stages over the years.

Then, in the summer of 1968, a new variant which was substantially different from all previous variants appeared in Hong Kong and caused extensive outbreaks in the Far East (Cockburn, Delon & Ferreira, 1969). This virus had an antigenically distinct haemagglutinin but its neuraminidase component was later shown to be similar to that of the earlier A2 strains (Coleman *et al.* 1968). The magnitude of the change in haemagglutinin, however, was such that antibody acquired by exposure to earlier variants was expected to confer little protection and widespread epidemics were forecast in all parts of the world, including Britain. This

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situation afforded an opportunity to study prospectively the spread of a new antigenic variant and the illnesses it caused in relation to serological measures of immunity and other factors thought to affect susceptibility to influenza virus infection and consequent illness. With the collaboration of the Royal Air Force Medical Service, an investigation was organized therefore to study the expected epidemic in the winter of 1968–9. In the event, no major epidemic occurred that winter. However, the following year, in 1969–70, there was a severe, explosive outbreak of influenza in all parts of the country. So it was decided to extend the study for a further year. Preliminary results have already been reported (Miller, Pereira & Clarke, 1971) and this paper gives the detailed findings.

MATERIALS AND METHODS

General plan

In the autumn of 1968 volunteers were recruited from among personnel in 13 R.A.F. stations in various parts of England and Wales. Blood specimens were obtained from the volunteers at that time and again in the spring of 1969. Any acute respiratory illness they reported in the intervening winter was investigated virologically and a clinical report was completed. All stations also kept records of every acute respiratory illness, in both volunteers and other personnel, which resulted in their absence from work.

After the unexpectedly severe epidemic in 1969–70 arrangements were made to extend the study by collecting a third blood specimen from all volunteers who could be contacted in the spring and summer of 1970, and to make a retrospective enquiry into any illness experienced in the previous winter.

Field methods

At the start of the study in October 1968, each station was visited by a doctor from the Epidemiological Research Laboratory, Colindale, who, with the help of the Station Medical Officer, enrolled the volunteers. A specimen of venous blood (5–10 ml.) was taken from each volunteer and a Personal Record Card was completed. This card recorded the following information: name of station, volunteer's name, rank, place of residence (i.e. billets, mess, married quarters), age and sex, blood group and influenza vaccination history (obtained from service or other medical documents), and current smoking habits with details of amount and type of tobacco smoked.

During the ensuing winter, when a volunteer reported to the Station Medical Officer with an acute respiratory or non-specific febrile illness, a nose and throat swab and acute and convalescent sera were obtained if possible. For each such illness a detailed clinical record was also completed and the date and diagnosis were entered on the Personal Record Card.

Copies of each station's weekly sickness returns (Stats. 58), which show details of all personnel absent from duty because of sickness for more than 48 hr., were sent to the Epidemiological Research Laboratory. From these returns weekly acute respiratory illness rates on each station were calculated.

Table 1. *The study population*

Blood specimens obtained	Total no. of persons	Illness records incomplete		Vaccinated		Total persons records complete and not vaccinated
		1968/9	1969/70	1968/9	1969/70	
1 and 2	912	18	—	119	—	775
2 and 3	560	—	19	—	62	479
1, 2 and 3	560	30		102		428

Stations were visited again in the early summer of 1969 and a second serum specimen was obtained from all volunteers who were still available. At the time of this visit a note was made of whether the volunteer had received influenza vaccine during the winter. Also, the survey records of illnesses were checked for discrepancies against each individual's service medical records and the Stats. 58. Few discrepancies were found, indicating that records were usually accurately maintained. If a volunteer was posted to another station in Britain during the winter, the Medical Officer at the new station was asked to continue the record of illnesses and, at the end of the winter, to take a second blood specimen and report whether or not the volunteer had been vaccinated against influenza. Volunteers who were posted abroad were not followed-up.

In the summer of 1970 an attempt was made to trace all volunteers from whom a second specimen of blood had been obtained the previous summer. This time those who had been posted abroad were included, but not those who had left the R.A.F. A third specimen of blood was taken from all those traced and the following details were entered on special record cards: the volunteer's account of his smoking habits immediately before the 1969–70 epidemic; whether or not he had received influenza vaccine since the second specimen of blood was taken; and all respiratory illnesses experienced in the interval which were recorded in the service medical records (i.e. illnesses for which the Medical Officer had been consulted). There was no means of checking the completeness of the records obtained, but cross-checks applied in the previous winter gave reason for confidence that omissions were likely to have been few.

Volunteer population

A total of 1183 volunteers, ranging from 30 to 136 on different stations, were recruited initially. Their average age was 29 years, with a range of from 17 to 57 years: 60% of them were under 30 years of age and 89% were male.

Subsequently, for a variety of reasons, many of the original volunteers were lost to the study; some because later specimens of blood were not obtained, some because they received influenza vaccine, and some because their illness records were not complete. The numbers whose records could be analysed, therefore, were depleted in successive stages of the study (Table 1). Of the original 1183 volunteers, 912 gave a second specimen of blood at the end of the first winter; but illness histories were incomplete for 18 of them and another 119 had received influenza vaccine during that winter. This left 775 volunteers for study during the winter

1968–9. A third specimen of blood was obtained from 560 of the 775; illness histories in the second winter were incomplete for 19 of these persons, and 62 received vaccine during that winter, leaving 479 for whom results could be analysed. Only 428 (36%) of the original 1183 volunteers gave three specimens of blood, had complete illness records throughout both winters and received no vaccine in either winter.

This lapse rate is high. However, we were unable to demonstrate any important differences between the base population and the residual group in respect of the distribution of the initial antibody titres, illness rates or other possibly significant variables about which we had information.

Laboratory methods

Routine blood specimens were transported by hand, or if this was not possible, by post, to the Virus Reference Laboratory, where the serum was separated and tested for haemagglutination inhibiting (HI) antibody by the methods described by Pereira, Pereira & Law (1964).

The initial sera, taken in October 1968, were examined on receipt for HI antibody to the A2/Singapore/57 virus and then stored at $-30°$ C. On receipt of the second sera, in the summer of 1969, the paired specimens were tested together for the presence of HI antibody to the Hong Kong variant of the A2 influenza virus, after which they were stored again at $-30°$ C. The third serum, taken in the summer of 1970, was tested for HI antibody in parallel with the first serum and, if a rise in antibody between the first and second sera had been detected, the third was also tested with the second serum. The initial sera from a selected group of volunteers (for convenience restricted to those from whom three specimens had been obtained) were also tested for HI antibody to A2/England/68 and A2/Tokyo/67 viruses which had been prevalent in the U.K. immediately before the Hong Kong/68 variant appeared and which carried the same neuraminidase as the A2/Hong Kong/68 virus.

The repeatability of antibody estimations was tested on a sample of specimens. No significant differences were found on repeated tests in the distribution of antibody titres between specimens or in the frequency of significant ($\geqslant$ fourfold) increases in titre.

The paired sera taken from volunteers when they became ill in the winter 1968–9 were sent to the nearest Public Health Laboratory for influenza complement-fixing antibody estimations (Bradstreet & Taylor, 1962). Swabs from the nose and throat were broken off into bottles containing a virus transport medium, placed on melting water ice and sent the same day to the laboratory, where they were examined for influenza virus by standard virus isolation procedures (Medical Research Council, 1965); all strains were sent to the Virus Reference Laboratory for further characterization.

Table 2. *Relation between diagnosis and laboratory results*

	Influenza	URTI	MRTI	LRTI	Total
Positive laboratory results*	14	6	1	1	22
Negative laboratory results	63	88	15	10	176
Total	77	94	16	11	198

* Influenza A virus isolated, or fourfold or greater increase in complement-fixing antibody titre.

RESULTS

The incidence of acute respiratory illnesses in the survey stations

The total population of the 13 R.A.F. stations which participated in the study was 14,710 in October 1968; the numbers on individual stations at this time ranged from 500 to 2900, though the figures varied during the study because of the movement of personnel. For this reason sickness rates have been calculated by taking man-weeks at risk as the denominator. The mean respiratory illness rates during the winter 1968–9 ranged from 2 to 5 per 1000 man-weeks in different stations. There were three peaks in the rates – in November, early January and the end of February; these peaks corresponded with those shown in the national morbidity statistics (Miller *et al.* 1971). The peak incidence of illnesses diagnosed clinically as influenza occurred at the same times as the peak incidence of other respiratory disease.

In the winter of 1969–70, when information was obtained restrospectively, records were received from only seven stations and for only part of the period in question. However, such records as were available confirmed that in these stations there was a large-scale influenza epidemic during December and January, which coincided with the national epidemic.

Illness rates in volunteers

The general pattern of illness during the two epidemics in volunteers was similar to that in all personnel on the same stations. During the first winter 38% of the volunteers observed had at least one respiratory illness; the rate for upper respiratory tract (URT) illnesses was 26%, for influenza 12% and for lower respiratory tract (LRT) illnesses 5%. In the second winter 41% had a respiratory illness; 28% had URT illnesses, 17% had influenza, and 8% LRT illnesses. Thus the attack rates for URT illnesses were similar in the two winters, whereas, those for influenza and LRT illnesses were proportionately considerably higher in the second winter than in the first.

In 1968–9 illnesses were investigated in the laboratory by examination of nose and throat swabs for virus and/or paired sera taken at the time of illness for a four fold or greater increase in titre of complement-fixing antibody. Table 2 shows the relation between the clinical diagnosis and laboratory results. Only 14 (7%) of the 198 illnesses investigated were diagnosed clinically as influenza and confirmed as such in the laboratory. These 14 comprised 64% of the 22 with positive laboratory results, but only 18% of the 77 illnesses called influenza.

Table 3. *Percentage distribution of HI antibody titres to four strains of A2 influenza virus in October* 1968

	Antibody titre					Total persons	
	< 1/10 %	1/10 %	1/20 %	1/40 %	⩾ 1/80 %	No.	%
A2/Singapore/57	10	5	9	17	58	559*	100
A2/Tokyo/67	40	16	14	15	16	554†	100
A2/England/68	46	13	16	12	13	554†	100
A2/Hong Kong/68	69	12	13	4	2	560	100

* Excludes one person whose A2/Singapore/57 antibody titre was not estimated.
† Excludes six persons whose A2/Tokyo/67 and A2/England/68 antibody titre was not estimated.

Table 4. *Relationship between titres of HI antibody to A2/Hong Kong/68 and A2/England/68 in October* 1968

Antibody titre to A2/England/68	Antibody titre to A2/HK/68					Total persons
	<1/10	1/10	1/20	1/40	⩾ 1/80	
< 1/10	**215**	**22**	15	3	0	255
1/10	**49**	**11**	8	1	1	70
1/20	50	**15**	**15**	**3**	6	89
1/40	32	9	**15**	**8**	**3**	67
⩾ 1/80	39	10	17	**5**	**2**	73
Total persons	385	67	70	20	12	554*

* Excludes six persons whose A2/England/68 antibody titre was not estimated.

Antibody distributions

The distribution of titres of antibody in the initial sera against selected variants of the influenza A2 virus are shown for the 560 persons from whom all three specimens were obtained (Table 3). Over 90 % of sera tested contained detectable antibody to the original 'Asian' A2/Singapore/1/57 virus and in the majority titres were high – 75 % being 1/40 or more. Antibody to variants prevalent more recently (A2/Tokyo/67 and A2/England/68) was less frequently present and the proportion of sera with high titres was much less than with the A2/Singapore/1/57 virus.

Antibody to the Hong Kong/68 variant was present in 31 % of specimens but most titres were low, only 6 % being 1/40 or more. It is now thought that these low titres of HI antibody were almost certainly directed, probably by the mechanism of steric hindrance, against the neuraminidase component of the variant, which is similar to that of the previously circulating variants, and not against the haemagglutinin which is antigenically quite distinct from that of earlier variants.

The relation between antibody to the Hong Kong/68 variant and that to the A2 strain prevalent in Britain the previous winter (A2/England/68) is shown in Table 4. The majority (76 %) of the 169 volunteers with detectable antibody to A2/Hong Kong/68 also had antibody to A2/England/68; of the 32 with high titres (⩾ 1/40) to A2/Hong Kong/68, 18 (56 %) had high titres to the earlier strain. In contrast, of the 385 without antibody to the Hong Kong strain a considerably

Table 5. *Percentage distribution of HI antibody titres to A2/HK/68 before and after the winters* 1968–9 *and* 1969–70

	Antibody titre					Total persons	
	< 1/10 %	1/10 %	1/20 %	1/40 %	⩾ 1/80 %	No.	%
October 1968	69	12	13	4	2	560	100
June 1969	56	11	9	5	19	560	100
June 1970	28	4	8	9	51	560	100

lower proportion (44 %) had detectable antibody against A2/England/68 and of these only 71 (18 %) had high titres against it. Conversely 40 (16 %) of the 255 persons without antibody to A2/England/68 had detectable antibody to the Hong Kong strain and only 3 (1 %) had titres ⩾ 1/40.

No relation was found between initial titre of antibody to A2/Hong Kong/68 and age or sex, ABO blood group, current smoking habits or history of influenza vaccination before October 1968. However, the proportion of volunteers who had antibody differed significantly between stations, with a range of from 18 to 58 %; these differences corresponded with differences between stations in the proportion of persons with antibody to A2/England/68 and were probably therefore related to whether or not there had been an epidemic of influenza on the station the previous winter.

The proportions of persons with different titres of antibody to A2/Hong Kong/68 before and after the two winters are shown in Table 5. In October 1968, 69 % had no detectable antibody and only 6 % had titres of 1/40 or more. However, after the first winter the proportion with no antibody had fallen to 56 % and after the second winter to only 28 %. Meanwhile, the proportion with high antibody titres (⩾ 1/40) increased to 24 % after the winter 1968–9 and to 60 % after the winter 1969–70.

Infection rates

Infection rates have been calculated on the assumption that, after excluding those who had been vaccinated, persons with a fourfold or greater rise in HI antibody to the A2/Hong Kong/68 virus between sera taken before and after either winter were infected with the virus in the relevant interval and those with no such rise were not. The infection rate in persons with antibody titres ⩽ 1/10 before the first winter was about 25 %; in the second winter the rate was more than double this figure (Table 6). Persons with higher initial antibody titres showed evidence of infection less often, and in those with antibody titres ⩾ 1/40 infection in either winter was infrequent.

Attention has been directed above to the frequent lack of correspondence between the clinical diagnosis of influenza and laboratory proof of infection at the time of illness. Table 7 shows that in both years only a small minority of persons with serological evidence of infection had an illness diagnosed as influenza, and, conversely, a substantial number were said to have had influenza in whom infection was not substantiated serologically. But during each winter about half of

Table 6. *Infection* rates in relation to initial antibody to influenza A2/Hong Kong/68 virus*

Initial antibody titre	Oct. 1968, no. of persons	Infected* 1968–9		Oct. 1969, no. of persons	Infected* 1969–70	
		No.	%		No.	%
< 1/10	475	121	25	269	155	58
1/10	108	27	25	55	30	55
1/20	113	21	19	41	10	24
1/40	50	5	10	27	3	11
⩾ 1/80	29	2	7	87	1	1
Total	775	176	23	479	199	42

* Fourfold or greater rise in HI antibody to A2/HK/68 in paired sera taken before and after each winter.

Table 7. *Illness rates in persons with and without infection**

Year		All persons		Persons with respiratory illness: Clinical Influenza		Persons with respiratory illness: Other respiratory		Persons without recorded illness	
		No.	%	No.	%	No.	%	No.	%
1968–9	Infected*	176	100	32	18	57	32	87	49
	Not infected*	599	100	48	8	145	24	406	68
1969–70	Infected*	199	100	50	25	47	24	102	51
	Not infected*	280	100	25	9	63	22	192	69

* Fourfold or greater rise in HI antibody to A2/Hong Kong/68 virus in paired sera taken before and after each winter.

those with serological evidence of influenza infection had *no recorded illness*, although it is possible that they had minor illnesses that were not reported.

There is some evidence from Table 7 that the diagnosis of influenza was more accurate in 1969–70 than in 1968–9. In each year just under one-quarter of those *without* evidence of infection experienced a respiratory illness to which a diagnosis other than influenza was attached. A similar proportion of such illnesses was reported in those *with* evidence of infection in 1969–70, but the proportion was higher (32%) in this group in 1968–9, suggesting that in that year it included some cases of influenza. More detailed analysis of the results for 1969–70 supports this suggestion (Table 8). This analysis, which excludes persons with more than one recorded illness during the winter, divides subjects into those who were ill during the height of the epidemic (December 1969 to January 1970) and those who were ill outside this period. The proportion of persons with clinical influenza who had evidence of infection was much higher during than outside the epidemic, and was nearly twice as high as in those with other respiratory diagnoses at any time. In contrast there was little difference in the frequency of infection between those with other diagnoses, either during or outside the epidemic, and those with no recorded respiratory illness.

Table 8. *Infection rates during the* 1969–70 *epidemic*

	Illness during epidemic		Illness outside epidemic		
	Influenza	Other resp.	Influenza	Other resp.	Not ill
Total persons*	44	26	13	58	294
Infected†					
No.	33	10	7	24	102
%	75	38	54	41	35

* Excludes persons with more than one illness.

† Fourfold or greater rise in HI antibody to A2/HK/68 in paired sera taken before and after each winter.

Initial antibody and illness rates

In the first winter, 1968–9, no clear relation could be demonstrated between the subjects' prior antibody titre against influenza A2/Hong Kong/68 virus and their susceptibility to respiratory illnesses, including influenza. However, the next year in 1969–70, there was a progressively lower illness rate with increased antibody titre (Table 9, col. 1). This relation was much stronger and can be seen in both winters if the analysis is restricted to persons with serological evidence of having had influenza infection during the winter (col. 2); the trend was significant in both years (1968–9, $P < 0{\cdot}05$; 1969–70, $P < 0{\cdot}001$). Of those with an antibody titre of 1/40 or more at the beginning of the winter, only three persons in 1968–9 and one person in 1969–70 had a record of respiratory illness accompanied by serological evidence of influenza. In both years non-influenza respiratory illness rates were higher in those with antibody than in those without (col. 3), possibly because the latter group experienced a high rate of infection with influenza virus (col. 2) which either interfered with other viruses or, by causing illness, reduced their chances of exposure to other viruses.

No correlation between the amount of tobacco smoked and respiratory illness rates could be demonstrated, whether there was serological evidence of infection or not, and whether the illness was diagnosed as influenza or not. There were too few attacks of lower respiratory illness for separate analysis.

Infection and illness rates in successive winters

Table 10 shows the relation between infection with or without illness in the first winter and infection with or without illness in the second winter.

There were altogether 158 persons who had one or more respiratory illnesses in 1968–9; 70 (44 %) of them had a respiratory illness the next winter compared with 89 (33 %) of the 270 who had not been ill the first year. Having a respiratory illness in 1968–9, therefore, gave no apparent protection against illness in 1969–70. However, of those who had *serological evidence of influenza infection* in 1968–9, whether or not they also had a reported illness, *none* was again infected in 1969–70. In contrast, of those who were *not infected* in 1968–9, 53 % were infected in 1969–70 and 26 % of them were ill.

Table 9. *Initial antibody and illness rates*

Year	Antibody titre to A2/HK/68 before winter	Persons		Attacks of respiratory illness: Total (1)		In persons with infection* (2)		In persons without infection* (3)	
		No.	%	No.	%	No.	%	No.	%
1968–9	< 10	475	100	201	42	72	15	129	27
	10	108	100	42	44	15	14	27	30
	20	113		55		16		39	
	40	50	100	17	37	1	4	16	33
	⩾ 80	29		12		2		10	
Total		775	100	327	42	106	14	221	29
1969–70	< 10	269	100	155	58	105	39	50	19
	10	55	100	21	44	12	18	9	26
	20	41		21		5		16	
	40	27	100	16	39	1	1	15	38
	⩾ 80	87		28		0		28	
Total		479	100	241	50	123	26	118	25

* Fourfold or greater rise in HI antibody to A2/HK/68 in paired sera taken before and after each winter.

Table 10. *Infection and illness rates in successive winters*

1968–9		Total No.	1969–70 Infected* Total No.	%	Infected* Ill No.	%	Not infected* Total No.	%	Not infected* Ill No.	%
Infected*	Ill	36	0	—	—	—	36	100	9	25
	Not ill	48	0	—	—	—	48	100	13	27
	Total	84	0	—	—	—	84	100	22	26
Not infected*	Ill	122	65	53	39	32	57	47	22	18
	Not ill	222	119	54	49	22	103	46	27	12
	Total	344	184	53	88	26	160	47	49	14

* Fourfold or greater rise in HI antibody to A2/HK/68 in paired sera taken before and after each winter.

It may also be noted that of the 122 persons who were ill but not infected in 1969–9, 61 (50 %) were ill again in 1969–70, compared with 76 (34 %) of the 222 who had not been ill the first year. Those who were infected in 1968–9 suffered a higher non-influenza illness rate (26 %) the next year than those who had not been infected (14 %), which partially offset the benefit derived from their immunity to influenza.

DISCUSSION

The unexpected pattern of the epidemics that followed the introduction of the A2/Hong Kong/68 influenza virus into Britain in 1968 and the contrast between its epidemiological behaviour in the winter 1968–9 and that in 1969–70 has been previously described (Miller *et al.* 1971). The introduction of a new antigenic variant into a susceptible population was expected to produce a major epidemic. In the event, although infection was widespread and the cumulative excess morbidity and mortality during the first winter was considerable, the impact of the virus was much less than had been forecast. The next winter, in 1969–70, an explosive epidemic such as had been expected the previous winter, occurred with dramatic effects on morbidity and mortality rates. The results of this serological study amplify the picture of the epidemics obtained from analysis of vital statistics.

At the start of the survey nearly all subjects had antibody to the original A2 virus (A2/Singapore/57), mostly at high titres, and more than half had antibody to recently prevalent variants in Britain. Less than one-third of the volunteers had detectable antibody to the new Hong Kong virus and in those who had it was usually at a low titre. Moreover, such antibody as was present was directed solely against the virus neuraminidase and not the haemagglutinin. It seems highly improbable that this pre-existing antibody was sufficient to have modified the epidemic in 1968–9, particularly since the next year a major epidemic was not averted despite the fact that the proportion of persons with antibody, in many cases at high titres, had increased from 31 to 44 %. By the end of the 1969–70 winter 60 % had antibody titres to the Hong Kong virus of 1/40 or more and for the

next two winters this virus gave relatively little trouble, whether because the proportion of immune persons was sufficient to prevent the spread of infection or for some other reason cannot be determined.

The extent to which antibody to one influenza virus variant protects against infection with succeeding variants and the significance of antibody in measuring protection against illness are important questions. The results of this study provide some information on these points. Infection (i.e. a fourfold or greater increase in antibody titre during a winter period) was observed much less often in those with high initial antibody titres than in those with low titres and was rare if the initial titre was 1/40 or more. Such protection against infection is useful only in so far as it equates with protection against illness. In the first winter no relation between antibody titres and the frequency of (total) respiratory illnesses was found, and in the second winter the reduction in illness rates in those with high titres was slight. However, if the analysis is restricted to those who showed evidence of influenza virus infection, the relation was pronounced in both winters, particularly the second, and statistically highly significant, even though some of the recorded illnesses may not have been influenza. A titre of 1/40 seemed to be the critical level at or above which illness coupled with evidence of influenza virus infection was rare. This finding is particularly important in relation to the results in the first winter since it implies that protection against illness was conferred by anti-neuraminidase antibody. These results indicate, therefore, that the presence of antibody can confer a measure of protection against illness that increases with higher titres. But proved infection with Hong Kong virus in the first winter appeared to confer absolute protection against infection with or without illness in the next winter. In contrast more than one-half of those who were not infected in the first winter were infected during the second, and one-quarter of them were ill.

The serological infection rate in the volunteers was nearly twice as high in the second winter as in the first, but the difference in respiratory illness rates was considerably less, even for illnesses diagnosed as influenza. The clinical diagnosis of influenza is notoriously difficult and the extent of diagnostic inaccuracy was illustrated by results from illnesses investigated in the first winter. There was some evidence, however, that when the epidemic was at its height during the second winter diagnostic accuracy was greater than at other times.

Our findings also suggest that symptomless infection is frequent since, in both winters about half the subjects who had evidence of infection had no recorded illness. It is possible that some illnesses were not recorded and others were too minor to call for medical attention, but it seems likely that many were symptomless. This finding is similar to that in the 1957 epidemics in Tecumseh (Hennessy *et al.* 1964).

The choice of servicemen for this study had disadvantages since their mode of life is not entirely representative of that of the majority of people in this country. Their opportunities for contact with one another, even in those who live outside the camp, are probably greater than in, for example, a group of factory workers who less often share leisure facilities. To offset this there are many advantages in the uniform and comprehensive medical facilities that are available for recording

illnesses and collecting appropriate blood samples. However, while it must be accepted that our results may not accurately reflect the situation in the population as a whole, the pattern of the epidemics in the Royal Air Force stations where the studies were carried out appears to have been similar to the experience of the rest of the population and the distribution of antibody in our subjects was similar to that observed in other population groups over the same period (Miller *et al.* 1971).

We are much indebted to the many Royal Air Force station medical officers, station commanders and their staffs, who gave much practical help with this study, and particularly to the volunteers for their patient co-operation. We also wish to thank Group Captains I. A. McIntosh and R. G. Covell for the arrangements they made in connexion with the study, the Directors of Public Health Laboratories who gave help with the collection and examination of specimens, and members of the staffs of the Epidemiological Research and Virus Reference Laboratories for their assistance. Finally, we acknowledge the permission of the Director General of Royal Air Force Medical Services to publish our findings.

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A bacteriological study of endemic tuberculosis in birds

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SUMMARY

Typing of *Mycobacterium avium* strains obtained in a study of endemic tuberculosis in a Wildfowl Reserve permitted the recognition of two separate infected groups. The main infection was in Anatidae and was due to *M. avium*, type 1; the other was in chickens used for incubation and brooding and the predominance in it of type 2 agreed with normal experience of birds, pigs and cattle in Britain. Many of the strains isolated from the Anatidae were aberrant and methods used to investigate these are described; two of the strains may belong to a new type. Birds which died from other causes, usually trauma, often had subclinical tuberculosis and 5% of the samples of mud and soil examined yielded *M. avium*.

INTRODUCTION

The Tuberculosis Reference Laboratory was recently invited by the Wildfowl Trust, Slimbridge, to study endemic tuberculosis occurring in their extensive collection of Anatidae (ducks, geese and swans). The causes of the endemic situation are being investigated and, it is hoped, progressively eliminated.

The results of a collaborative investigation into the bacteriological aspects of the subject were unexpected in some respects and are recorded here as an aid to further studies of a similar nature. Two series were examined of birds found dead or moribund and considered tuberculous, the main series comprising Anatidae, the other chickens used for incubation and brooding. A third series consisted of Anatidae found dead, usually from trauma, and judged then to be free from tuberculosis. In each series the preliminary diagnosis was made by macroscopic examination of the viscera and Ziehl–Neelsen films. Finally, a study was made of samples of soil, mud and muddy water from the environment of the birds.

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MATERIALS AND METHODS

The birds were refrigerated until necropsy was possible and a portion of liver was then sent to the Reference Laboratory by post. A further piece was fixed by formalin for section but as this procedure did not prove to be of value it was discontinued after 16 cases.

Microscopical examination

Liver samples were homogenized by grinding in distilled water. The suspensions obtained after coarse fragments had settled were centrifuged (1200 *g* for 20 min.) and films of the deposits made for staining by the Ziehl–Neelsen method. They were graded as scanty, moderate or heavy positives or as negative. Environmental specimens were not subjected to microscopy.

Cultural examination

The centrifuged deposits used for films were each mixed with 2 ml. 5% H_2SO_4 (v/v). Each mixture was transferred to a sterile bottle and held at room temperature for 40 min. It was then diluted with 18 ml. sterile water, centrifuged for 20 min. at 1200 *g* and the deposit obtained after decantation inoculated on two slopes of Lowenstein–Jensen medium in universal containers and into Kirchner medium. Penicillin, 100 units/ml., was included in the Kirchner medium and 60 units/ml. in one of the two slopes.

Soil and mud specimens were shaken mechanically with sterile water and allowed, as were shaken specimens of muddy water, to settle at 4° C. until the supernatant fluid was only faintly turbid (2–4 days). To two 10 ml. volumes of this fluid were added 10 ml. of (*a*) 0·26% and (*b*) 0·06% benzalkonium chloride respectively, each in 10% trisodium phosphate (Jones & Jenkins, 1965). The mixtures were shaken occasionally during 60 min. at room temperature, centrifuged for 20 min. at 1200 *g* and decanted. The deposit was neutralized to phenol red with 0·1 N-HCl and inoculated on Lowenstein–Jensen medium with and without penicillin. The remainder of each specimen was held at 4° C. and if the cultures became contaminated, 2 ml. volumes of similar suspensions were each treated with 2 ml. N-NaOH for 15, 30 and 60 min. respectively in a 37° C. incubator, diluted and centrifuged. The deposits were each inoculated on two slopes of buffered egg medium containing penicillin.

All inoculated media were incubated at 37° C. The slopes were discarded after 6 weeks if negative, the Kirchner media then being checked by subculture on egg medium.

Identification of strains

A limited systematic examination was carried out comprising morphology, the character of growth at 25, 37, 42 and 45° C., pigmentation after incubation in light and in the dark at 37° C., arylsulphatase activity and sensitivity tests on cycloserine and ethionamide. The methods used for these tests were described in detail by Birn *et al.* (1967).

Except for the fast-growing mycobacteria cultured from the environment, all strains were subjected to lipid analysis by thin-layer chromatography as described by Marks, Jenkins & Schaefer (1971). Known strains of *M. avium*, types 1 and 2, were examined in parallel on each plate.

All the strains subjected to lipid analysis were typed by agglutination and if necessary by absorption of agglutinating sera. The serological methods used and the preliminary plating for homogeneity were described by Schaefer (1965).

Passage of aberrant strains

Sixteen strains isolated from Anatidae differed initially in one or more respects from what was regarded as the normal behaviour of *M. avium* (see Results). Each was inoculated intravenously into two fowls at a dose of 0·01 mg. Survivors were killed after approximately 4 months. The liver and spleen of these and of the birds which died were examined histologically and for mycobacteria by microscopy of smears and sections and by culture. The 64 re-isolated strains obtained were subjected to the systematic examination, lipid analysis and serological tests used for the original cultures. Pigmented strains were plated at high dilutions to detect mixtures of organisms – initially on egg medium, but if they still appeared pure a single colony was further plated on oleic acid–albumin agar medium in the manner of Schaefer (1965).

RESULTS

The main investigation consisted of the examination of 78 Anatidae found dead or moribund in the course of 15 months which were considered to be tuberculous on macroscopic examination of the viscera. The results of the cultural examinations made and the classification of the isolates are summarized in Table 1. One culture was lost by contamination. A reasonable assessment of the error of macroscopic diagnosis is provided by the outcome of negative cultures in eight cases – six being negative on filming and two scanty positives. In addition, one of the positive cultures was probably not significant, the film having been a scanty positive and the organism *M. intracellulare* (B 31). Of the other 68 cultures, 55 were finally identified as *M. avium* type 1, eight as *M. avium* type 2, and five were unclassifiable. All the latter had the cultural characters of *M. avium* but lacked the lipids or antigens of known types; two were virulent and the group therefore may contain or comprise a new entity. In one of the virulent strains a serological and lipid relationship to type 3 was suspected on first isolation but not confirmed.

M. avium was isolated from 10 of the 21 dead Anatidae considered non-tuberculous at necropsy but growth was usually scanty and microscopy was negative in six cases. The findings suggest that in most such cases the organism was not causing serious infection.

The tuberculosis in chickens had evidently an independent epidemiology because, of the strains of *M. avium* isolated, eight belonged to type 2, three to type 3 and only one to type 1, a distribution quite different from that found in the Anatidae.

Two strains of *M. avium* type 2 and one of type 1 were isolated from 65 environ-

Table 1. *The isolation of* M. avium *and other mycobacteria from the livers of birds found dead or dying in a Wildfowl Reserve and from their environment*

Series	Results of culture with the number and character of the strains isolated	
Anatidae considered to be tuberculous at necropsy	*M. avium* type 1	48
	M. avium type 2	5
	Negative on culture*	8
	Contaminated	1
	16 aberrant strains; final diagnosis:	
	M. avium type 1	7
	M. avium type 2	3
	M. intracellulare	1
	Uncertain status	5
	Total	78
Anatidae considered not to be tuberculous at necropsy	*M. avium* type 1†	8
	M. avium type 2‡	2
	Negative on culture	11
	Total	21
Chickens used for fostering and considered tuberculous at necropsy	*M. avium* type 1	1
	M. avium type 2	8
	M. avium type 3	3
	Total	12
Soil, mud or muddy water from the Anatidae environment	*M. avium* type 1	1
	M. avium type 2	2
	Free-living mycobacteria	14
	Negative on culture	47
	Contaminated	1
	Total	65

* Six liver homogenates were negative on microscopy at the Reference Laboratory (Ziehl–Neelsen stain) and two were scanty positives.

† Five homogenates negative similarly, three positive. One strain was aberrant, being serologically type 1 but without specific lipid.

‡ One negative similarly, one positive.

mental specimens. As only a small proportion of the considerable area of the Wildfowl Reserve was sampled, the positive findings probably represent a large reservoir of infective material.

Aberrant strains

Of the 69 strains considered to be possibly *M. avium* which were isolated from the main series of Anatid birds, 16 were aberrant in at least one character. In contrast, none of the 12 chicken or 3 environment strains was aberrant. One was aberrant of the 10 strains isolated from Anatidae considered non-tuberculous on macroscopic examination. Each of the aberrant strains was inoculated into two fowls, and when they died or were killed cultures were obtained from spleen and liver. Details of the characters of the original and passaged strains from the main series are given in Table 2. In these 16 cases the final diagnosis was *M. intracellulare* in one and *M. avium* of unknown or uncertain type in five. Ten strains were finally assigned to *M. avium* type 1 or type 2 and in five of these the diagnosis of type would not have been reached without the passage. It appears therefore that when

a strain is rough and lacks specific lipid on initial examination, a second attempt may be successful and save the use of passage. Re-examination will be more effective if the strain is first plated on oleic acid–albumin agar medium and a transparent colony selected. In the case of six typed *M. avium* strains, only one of the two fowls inoculated died in the 4 months observation period and in two other cases (B 23, B 94), neither fowl died. These results illustrate the limitations of a virulence test in the diagnosis of *M. avium* when there is a tendency to roughness.

Five of the unclassified strains, B 19, B 46, B 98, B 120 and B 122, had the cultural characters of *M. avium* but lacked specific lipid and could not be identified serologically. Two of these, B 19 and B 46, each killed the two fowls inoculated but the other three were avirulent. A weak lipid pattern suggestive of *M. avium* type 3 was observed in the initial analysis of strain B 46 and a trace of agglutination obtained with type 3 serum, but these findings were not confirmed in repeat tests, nor was any type-specific character detected after passage.

Three of the primary isolates from birds were pigmented. One proved to be a pure strain of *M. avium* type 1. The second was *M. intracellulare* and the third was a mixture of nonpigmented *M. avium* type 2 and pigmented *M. intracellulare*. The mixture persisted after passage and could not be separated on egg medium although plating on oleic acid–albumin agar medium was successful.

DISCUSSION

The present investigation demonstrates that classification at type level is needed to understand the epidemiology of avian tuberculosis. Its use was essential to the recognition of two separate endemic infections in the Wildfowl Reserve studied. In the Anatidae, 87% of the typable strains of *M. avium* isolated belonged to type 1 compared with only 8% of the strains from tuberculous chickens in the same unit. The distribution of types in the chickens was very similar to that met in unselected sporadic isolates from wild, game and zoo birds and fowls examined in the Reference Laboratory and at Weybridge in recent years (Table 3).

The Reference Laboratory's experience of strains from British Anatidae outside the Wildfowl Reserve totals nine (four since series A), of which three were type 1, five type 2 and one type 3. The two most recent were pigmented type 1 strains isolated from ducks from different counties. Gordon, Garside, Dobson & Reid (1941) record the isolation of rough strains from ducks but it now appears that other aberrancies occur with some frequency amongst Anatidae strains. Three other examples of Anatidae infected with pigmented type 1 *M. avium* have been recorded (Chalquest & Matsuoka, 1962; Schaefer, 1965) making a total of six with those described here. We have also been informed of a similar isolation from a cormorant, which suggests an association between pigmentation and water as a medium of infection.

A striking feature of the present investigation was the difficulty or failure met in typing 14 of the 68 strains in the main series considered to be *M. avium*. The difficulty occurred in lipid analysis in one case, serotyping in another and both techniques together in 12 cases. This experience contrasts with the immediate typing by lipid analysis of all 70 sporadic and chicken isolates examined by the

Table 2. *Examination of* 16 *strains which were not typical* M. avium *on primary isolation*

No.	Aberrant characters on primary isolation	Survival time of 2 inoculated fowls*	Properties after passage through fowls: Serology	Properties after passage through fowls: Lipid pattern	Conclusions about the nature of the organism
B 15	Rough;† spontaneous agglutination	(*a*) 6 weeks (*b*) Survived	*M. avium* type 1	*M. avium* type 1‡	A rough *M. avium* type 1. Smoothness (i.e. proportion of smooth colonies) was improved by passage
B 19	Rough; spontaneous agglutination. No specific lipid	(*a*) 6 weeks (*b*) 10 weeks	All rough; either spont. agglut. or unclassified§	Non-specific	In the absence of recognized type characters, the virulence suggests a new type or variety of *M. avium*
B 23	Rough.‖ No specific specific lipid	(*a*) Survived (*b*) Survived	*M. avium* type 1	*M. avium* type 1¶	A rough *M. avium* type 1 of low virulence. Smoothness was still limited after passage
B 31	Serologically unclassified.§ No specific lipid. Pigmented	(*a*) Survived (*b*) Survived	1 isolate unclass.§ 3 isolates rough and spontaneously agglutinated	Non-specific	*M. intracellulare*. The absence of virulence and of any specific property weighed against *M. avium* which also is hardly ever pigmented
B 38	Pigmented	(*a*) 6 weeks (*b*) 7 weeks	*M. avium* type 1	*M. avium* type 1	A pigmented *M. avium* type 1. Purity established by repeated plating
B 40	Rough; serologically unclassified.§ No specific lipid	(*a*) 8 weeks (*b*) Survived	*M. avium* type 2	(1) *M. avium* type 2. (2) Non-specific (from survivor)	A rough *M. avium*, typable only after passage. Smooth colonies were too few in the second case to give the specific lipid pattern
B 46	Rough (trace of type 3 agglutination). Weak? type 3 lipid pattern	(*a*) 8 weeks (*b*) 8 weeks	Rough and unclassified§	Non-specific	Either a new virulent type of *M. avium* or a variety of type 3. Only rough colonies were found on plating passaged isolates
B 47	Rough; unclassified.§ No *Avium* lipid pattern. Pigmented	(*a*) 10 weeks (*b*) Moribund at 16 weeks	*M. avium* type 2 mixed with rough *M. intracellulare*	*M. avium* type 2 mixed with *M. intracellulare*	The separation of *M. avium* and the pigmented *M. intracellulare* was only achieved by plating on oleic acid–albumin agar medium
B 59	No specific lipid (*M. avium* type 1 on repeat)	(*a*) 6 weeks (*b*) Survived	*M. avium* type 1	*M. avium* type 1	A rough *M. avium* type 1. The use of passage could have been avoided (see text)

Table 2 (*cont.*)

No.	Aberrant characters on primary isolation	Survival time of 2 inoculated fowls*	Properties after passage through fowls: Serology	Properties after passage through fowls: Lipid pattern	Conclusions about the nature of the organism
B 94	Rough; spontaneous agglutination. No specific lipid	(*a*) Survived (*b*) Survived	*M. avium* type 1	*M. avium* type 1	A rough *M. avium* type 1. Even after passage smooth colonies were few; one isolate was all rough and lacked specific lipid
B 98	Rough; spontaneous agglutination. No specific lipid	(*a*) Survived (*b*) Survived	Rough; spontaneous agglutination	Non-specific	Cultural characters of *M. avium* but no support from pathogenicity, serology or lipid analysis
B 106	Rough (a few *Avium* 2 colonies on repeat). No specific lipid. (*Avium* 2 on repeat)	(*a*) 10 weeks (*b*) Survived	3 isolates rough. 1 *M. avium* type 2 after re-examination	*M. avium* type 2	A rough *M. avium* type 2. If the repeat tests had been awaited passage would have been unnecessary
B 107	Rough. No specific lipid	(*a*) 14 weeks (*b*) Survived	3 isolates rough. 1 *M. avium* type 1	*M. avium* type 1 (1 isolate non-specific)	A rough *M. avium* type 1. Smoothness was still limited after passage
B 113	Rough. No specific lipid	(*a*) 12 weeks (*b*) Survived	3 isolates rough. 1 *M. avium* type 1 (from survivor)	Non-specific	A rough *M. avium* type 1. Only one isolate could be identified after passage
B 120	Rough. No specific lipid	(*a*) Survived (*b*) Survived	Rough	Non-specific	As B 98
B 122	Rough; unclassified.§ No *Avium* lipid	(*a*) Survived (*b*) Survived	Rough	Non-specific	As B 98

Rough strains could not be typed by sero-agglutination except in cases where this is indicated.
* Survivors killed after 4 months.
† *M. avium* type 1 serum reduced to half of original titre by absorption.
‡ Same result with primary isolate.
§ No agglutination with any *Avium-intracellulare* serum held.
‖ *M. avium* type 1 serum reduced to 1/16 of original titre by absorption.
¶ With two isolates the preliminary selection of a smooth colony was necessary.

Table 3. *Comparison of the general incidence of* M. avium *types with the Wildfowl Reserve incidence*

	M. avium types		
	1	2	3
A. Unselected isolates from birds			
(1) Reference Laboratory	7	38	11
(2) Weybridge	7	37	8
B. Wildfowl Reserve, chicken cases	1	8	3
C. Wildfowl Reserve, Anatidae cases	55	8	0

Reference Laboratory and their successful serotyping in all 53 cases attempted with only the need for two repeat tests. The findings may reflect a general association of aberrant strains of *M. avium* with tuberculosis in Anatidae but was probably mainly due to a variation or tendency of the endemic type 1 organism towards roughness and loss of specific lipid; this extended well beyond the 14 difficult strains. The variation may perhaps have been caused by infection of or relapse in birds which had residual immunity from previous experience of that type. The isolation of *M. avium* from several birds lacking evidence of overt tuberculosis indicated the prevalence of subclinical infection which would result in such immunity.

Two of the *M. avium* strains met in the present study had the cultural characters of *M. avium* and were highly virulent for fowls but could not be typed by present methods. One gave a trace of agglutination with type 3 serum and initially a weak lipid spot of the type 3 pattern but these findings could not be confirmed on repetition or with the four isolates obtained after passage. It must be left for further investigation to decide whether these two virulent strains represent a new type or variants of a known type as described by Schaefer, Davis & Cohn (1970).

As a result of the experience gained it can now be seen that much labour will be saved in the typing of difficult *M. avium* strains by first plating on oleic acid–albumin agar medium. The selection of a transparent colony for the examination will give the best chance of successful typing by agglutination or lipid analysis. The same procedure should be applied to pigmented strains in order to detect whether a mixture is present; egg medium appears to be unsatisfactory for this purpose and passage may fail to separate the organism (case B 47). Finally, if typing remains impossible, passage may be considered. At least two fowls should be inoculated and at least two different organs cultured at necropsy. It may be necessary to plate the cultures obtained again to find suitable colonies.

Although the present study does not embrace the basic causes of the endemic situation in the Reserve, the recovery of three strains of *M. avium* in its limited examination of the environment appears to be significant. Resistance to tuberculosis may also be affected by the protein and vitamin content of the food supply (Ratcliffe, 1946).

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A bacteriological evaluation of laminar-flow systems for orthopaedic surgery

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SUMMARY

An evaluation has been undertaken of the efficiency of laminar-flow ventilation in operating-rooms in which conventional operating-room clothing was used. It has been demonstrated that velocities in the region of 0·3–0·4 m./sec. will give maximum returns for effort in both down-flow and cross-flow systems. At this velocity the laminar-flow system, in terms of airborne bacteria measured at the wound site, was about 11 times more efficient using horizontal air-flow and 35–90 times more efficient using vertical air-flow than a plenum-ventilated operating-room.

INTRODUCTION

An ever-present problem in hospitals is the control of hospital infection, and in spite of much medical research in the past few decades wound sepsis remains one of the chief hazards.

There is, however, reasonable evidence that in general surgery some reduction in wound infection is obtained by positive-pressure ventilation of conventional operating rooms (Blowers, Mason, Wallace & Walton, 1955; Shooter, Taylor, Ellis & Ross, 1956). Recently, however, systems have been evolved which use large quantities of sterile air introduced through an entire end wall or ceiling in a unidirectional manner at velocities of up to 0·5 m./sec. (100 ft./min.) (Scott, 1970). These are normally known as laminar-flow systems.

Although there is no convincing evidence to show that this type of ventilation will result in a reduction of wound sepsis, it would seem reasonable that in 'clean' operations of long duration with implant of foreign material, as for example in a total prosthetic replacement of the hip, some reduction in wound sepsis should be possible. In a previous article (Whyte, Shaw & Barnes, 1971) we described the construction of an experimental operating-room with laminar-flow ventilation. This has now been subjected to a bacteriological evaluation which is described in this paper.

EXPERIMENTAL DESIGN

The experimental laminar-flow system was installed in one room of a twin-theatre suite which was built during the last war and solely served the Professorial Orthopaedic Unit of Glasgow University. Various types of elective orthopaedic surgery were carried out but we confined ourselves to observations on operations of the spine and total prosthetic replacement of the hip and knee.

One of the theatres was of conventional design, 0·7 $m.^3$/sec. (1500 c.f.m.) of heated, filtered air being supplied to the operating-room through four ceiling diffusers. The other identical theatre contained our laminar-flow unit and was served by the same staff and provided with the same instruments, clothing, etc. The sterilizing room was situated between the two theatres.

The experimental ventilation unit was built so that during an operation the direction of air could be changed from cross-flow to down-flow and the speed of the air could be varied in the case of the cross-flow from 0 to 0·6 m./sec. (120 ft./min.) and from 0 to 0·5 m./sec. (100 ft./min.) with the down-flow.

Measurement of the velocity was by a hot-wire anemometer placed 1 m. away from the filter face. If an obstruction influenced this measurement, velocity readings were calculated from the velocity of air issuing from the system.

Samples of air were taken from the vicinity of the wound, rather than the theatre environment, as the latter may bear no relationship to the risk of air-borne contamination. It may be considered that it would have been best to expose agar settle plates in order to obtain a direct indication as to the number of bacterial particles which would fall into the wound. This we did on a limited number of occasions, but even with the use of a large diameter Petri dish (15 cm.) the sample of bacteria was too low (about 2 bacterial particles/plate/hr.) to obtain significant results in a satisfactory time. We therefore used a High-Volume Slit-Sampler (Casella Ltd., London) with an air flow of 700 l./min. (25 c.f.m.). This was connected by a 0·6 m. (2 ft.) long bend of 10 cm. diameter (4 in.) flexible ducting to a metal cone 43 cm. (17 in.) long. This cone, which was sterilized between operations, was constructed to converge to a hole of 4 cm. diameter (1·5 in.) through which a sample was taken at a maximum of 15 cm. (6 in.) from the wound site.

Because of the very large sample of air around the wound (25 $ft.^3$/min. or 700 l./min.) and the fact that the sampling point was halfway along the incision and below it, we felt that good sampling conditions were established. Bacterial samples were incubated for 36 hr. at 37° C. before counting.

Samples were also taken, when appropriate, of dust particles 0·5 μm. and over. This was done by means of a Royco Particle Counter, the sterile tube being clipped to the sampling cone and samples taken at the same spot as the bacterial sample. It was considered that as very few dust particles would pass through the high-efficiency filters, any dust particles collected would normally be generated by potential sources of contamination, i.e. by the operating team.

The operating personnel were of course aware of the purpose of these studies and attempts were made at the start to place the team in the best position for good airflow. This was not pursued, however, during the observations reported here,

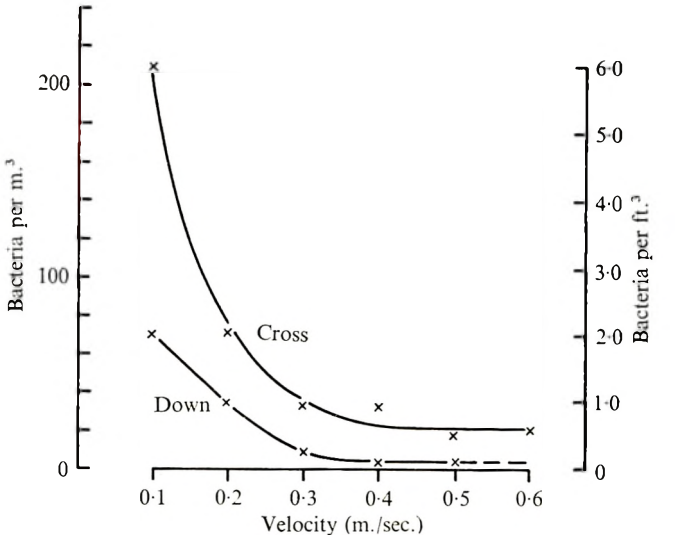

Fig. 1. Airborne bacterial counts at different velocities under cross- and down-flow conditions.

and positions were taken up which were more suitable to surgery. Although discipline was enforced by the physical shape of the system and the position of the anaesthetist at the end away from the filter, no artificial positions were adopted. All the surgery was done in a standing position and the clothing was of a conventional type; that is to say, it was of 'balloon cloth' which is known to do little to prevent the dispersion of bacteria from personnel.

RESULTS

Comparison of down-flow with cross-flow and the effect of velocity

Bacterial counts

Fig. 1 shows the average bacterial concentration that was found at the wound site at different air velocities under both down-flow and cross-flow conditions.

These results were obtained by sampling during 20 operations under cross-flow (13 hips, 5 spines and 2 knees) and 16 operations under down-flow (10 hips, 4 spines, 2 knees). Depending on the expected concentrations, samples were taken for 7–10 min. at a given velocity; in all a total of 270. The velocity was then randomly changed and another sample taken. This meant that the range of velocities was covered at least once during an operation. It may be seen that, at all velocities, down-flow ventilation gives a lower average concentration of bacteria than cross-flow. The difference in concentration was statistically significant at an F-level of 0·1 % at all velocities except 0·2 m./sec. where it was significant at 1 %.

Particle counts

Particle counts were taken at 1 min. intervals during the time the bacterial sampler was running. When, however, diathermy or a high-speed saw was being used, as well as when swabs were vigorously shaken, the particle counts increased

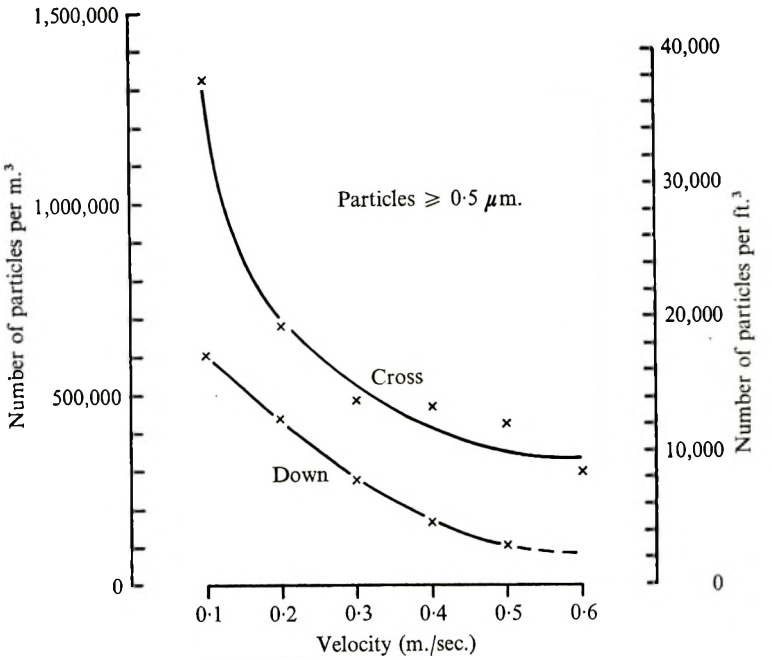

Fig. 2. Dust-particle counts ($\geqslant 0{\cdot}5\ \mu$m) at different velocities under cross- and down-flow.

tremendously. These counts were removed from the calculations and the counts averaged. The particle counts were not taken during as many operations as bacterial counts, but 18 operations were studied.

Fig. 2 shows the count of dust particles of 0·5 μm. or greater under down-flow and cross-flow conditions at different velocities. It may be seen that substantially the same graph is obtained from dust particles as bacterial particles.

Comparison of the laminar-flow system with a conventionally ventilated operating room

Samples of dust particles and bacteria were taken at the wound site in the conventional room using the same methods as described above. Bacterial samples were taken for a much shorter period (1 or 2 min.) because of the higher concentration in the air and hence a much larger number of samples could be taken during the operation. A total of 86 samples was taken. Ten operations were monitored (6 hips and 4 spines) and the average bacterial count in the theatre was found to be 350/m.³ (9·8/ft.³). The average bacterial count for each operation varied from 165 bacteria/m.³ (4·7/ft.³) to 665/m.³ (19·0/ft³.), individual samples varying from 35 bacteria/m.³ (1·0/ft.³) to 880/m.³ (25/ft.³). The average number of people in the operating room during these tests was eight.

Dust particle counts in the conventional theatre were in the region of 1,750,000–14,000,000 particles/m.³ (50,000–400,000/ft.³) for particles $\geqslant 0{\cdot}5\ \mu$m. (average 5,250,000/m.³ or 150,000/ft.³).

A comparison of the airborne count in the conventional operating room as

Table 1. *Percentage reduction of bacterial and particle count at different velocities of laminar-flow compared with conventional ventilation*

		Air velocity (m./sec.)					
		0·1	0·2	0·3	0·4	0·5	0·6
Bacteria	Downflow	79·5	90·0	97·1	98·9	98·8	—
	Crossflow	39·0	79·4	90·2	90·5	94·6	93·9
Particles	Downflow	88·6	91·7	94·8	96·9	98·0	—
$\geqslant 0{\cdot}5$ μm	Crossflow	74·8	87·1	90·1	91·1	92·0	94·5

opposed to the laminar-flow one is dependent on the velocity at which the laminar flow was being run. Table 1 is drawn up to show this comparison.

These results show that a velocity of 0·3 m./sec. (60 ft./min.) would reduce the bacterial airborne count by 97% using a down-flow of air and 90% using a cross-flow. Increasing the air velocity above this figure is accompanied by further reductions but these are small in comparison to the initial reduction achieved by a velocity of 0·3 m./sec. (60 ft./min.). Very similar results are achieved in reducing the dust particle counts.

DISCUSSION

Few systematic evaluations of the use of laminar-flow systems in operating theatres have been done, and the advice and results obtained by people involved in the industrial field are at present being used to build laminar-flow systems. What we have attempted to do is to evaluate laminar flow in such a way that systems may be designed for operating rooms to a predicted performance.

As in so many situations, the effort (and hence costs) expended in achieving very low airborne bacterial counts in an operating room is not proportional to results. This means that a point must be drawn where moderate effort and good design will achieve a highly efficient system. We consider such a point exists with an air velocity in laminar-flow systems of 0·3 m./sec. (60 ft./min.). An increase in velocity to 0·4 m./sec. (80 ft./min.) would give an additional margin of safety. At this speed 0·3–0·4 m./sec. (60–80 ft./min.) one would achieve a reduction in bacterial count of around 90% with the cross-flow and between 97–99% with the down-flow. It is accepted industrial practice (Federal Standard No. 209*a*) that the air velocity should be 0·46 m./sec. (95 ft./min.). The use of this recommended speed of between 0·3 and 0·4 m./sec. (60–80 ft./min.) will ensure a quieter system which will be considerably less expensive in capital and running costs.

It may be seen from the above results that in orthopaedic surgery (and almost certainly in most other fields of surgery) the down-flow system appears to be bacteriologically superior to that of cross-flow ventilation.

At velocities of 0·3, 0·4 and 0·5 m./sec. (60, 80 and 100 ft./min.) the down-flow ventilation had 3·5, 9 and 4·5 times less airborne bacteria respectively than cross-flow ventilation. In many ways this reduction is not the result we would have preferred as the design and operation of the cross-flow system has much to encourage its use. With a prefabricated system it is in general cheaper to build and install, and conventional lighting and services may be used without alteration. The

down-flow system is more difficult for the operating team to work with owing to the lack of accessibility caused by the necessity of four sides and often requires a larger surface area of filters. Most of these objections will no doubt be overcome as the technology of building ultra-clean operating rooms becomes more advanced. There will no doubt be compelling reasons for the adoption of one system or the other, but we hope that, with the data provided above, the choice of design of the system may be put on a rational basis.

It is also worth recording that these differences exist when conventional clothing is used. Use of impervious clothes may reduce this difference to practically nil.

We should like to thank Mr J. T. Brown, Mr J. Graham and Mr. S. K. Wood for their co-operation during this study as well as that of Sister Doherty and the theatre staff. Our thanks must also be given to Mr P. Bailey for his skilled technical assistance.

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The fate of a toxigenic strain of *Staphylococcus aureus* in vacuum-packaged bacon

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SUMMARY

Pork was cured by (*a*) the Wiltshire method and (*b*) a hygienic sweet cure process. Representative samples of both bacons were inoculated at 'low' density (10^3 organisms/g.) and 'high' density (10^6 organisms/g.) with a toxin-producing strain of *Staphylococcus aureus*, 'High' and 'low' density samples of both bacons were each stored at 5° C. for 42 days and 15° C. for 21 days.

Results indicated that the test organism at high inoculum density grew slowly in both bacons at 5° C. The organism survived at 5° C. in both 'low density' bacons. At 15° C. the test organism grew; growth being more pronounced in the 'hygienic' than in Wiltshire bacon.

INTRODUCTION

Previous work has shown that the shelf life of packaged bacon is influenced by, among other factors, the bacterial load at the point of packaging and the temperature of subsequent storage (Dempster, 1972). To ensure a satisfactory shelf life every effort must therefore be made to reduce initial bacterial numbers to a minimum. However, reducing the initial bacteriological count may, inadvertently, introduce a further hazard, namely the growth of potentially pathogenic organisms, particularly coagulase-positive staphylococci (CPS). Ingram (1962) considered that pathogens are hindered from growing in vacuum-packed bacon by what he called the 'population pressure' of an already established microflora. Thus he showed that staphylococci introduced into anaerobic packs of normal bacon at a concentration of 10^3 per g. increased to 10^4 to 10^5 per g. when the indigenous flora was first drastically reduced by irradiation. He concluded: 'accordingly in so far as the method of packing weakens the spoilage flora more than the pathogen it seems possible that packaging might conceivably encourage the more frequent survival of the latter'. By improving hygiene in the manufacture of packaged bacon and thereby reducing the number of spoilage organisms, accidental contamination of the product with CPS might therefore result in their multiplication. Furthermore, Ingram (1960) considered that a warm vacuum pack of bacon might act as an unintentional selective medium for staphylococci since

they can reduce nitrate, are proteolytic, facultatively anaerobic and have a temperature optimum for growth near 37° C.

Despite Ingram's (1960) suggestion, vacuum packaging *per se* has been shown by Christiansen & Foster (1965) to be inhibitory. They reported that *Staphylococcus aureus* increased about six generations on sliced ham at both 20° and 15° C. under vacuum, but went through 11–12 generations in the absence of vacuum and reached numbers which were 20-fold greater than the maximum populations which developed under the anaerobic conditions of a vacuum pack.

However, results of particular investigations have shown that staphylococci have grown at < 10° C. Thus Angelotti, Foter & Lewis (1961) found food-poisoning strains grew well in custard at temperatures from 5° to 45° C. Ginsberg (1945) studied the effects of immersion in commercial bacon brines on meat inoculated with various organisms including *Staph. aureus* and reported that this latter organism survived but did not multiply for 21 days at 8° C. Similarly Buttiaux & Moriamez (1958) observed that *Staph. aureus* survived in curing brines for 16 days at 6° C. although numbers decreased slowly over this period. Bardsley & Taylor (1960) reported a slight initial rise (up to 24 hr.) in staphylococcal count in Danish smoked bacon which was vacuum packed and stored at 5° C. They attributed this to growth during the initial stages of the experiment before the bacon was cooled to the holding temperature. However, they found that over 44 days staphylococci decreased only from about $10^{6\cdot8}$ to about $10^{5\cdot5}$ per g., that is, about 80·0 %. According to Bryan (1968), for a staphylococcal intoxication to occur, there must be (*a*) a reservoir of *Staphylococcus aureus*, (*b*) a mode of dissemination of the organisms, (*c*) contamination of a food that is capable of supporting the growth of the organism, (*d*) a suitable temperature for such time as to allow adequate multiplication and toxin production, and (*e*) consumption of sufficient amounts of toxin by a susceptible person.

In view of these comments it was thought desirable to study the fate of a toxin-producing strain of *Staphylococcus aureus* after its introduction to packs of sliced bacon. Although a considerable quantity of vacuum-packaged bacon is still produced from Wiltshire-cured middles, an increasing amount is made from pork cured in short-time (48 hr.) sugar brines. This product is usually produced and stored in the factory under refrigeration (5° C.) but may be exposed to higher temperatures during distribution. Commercially produced packaged bacon may be contaminated with variable numbers of staphylococci.

METHODS AND MATERIALS

To study the influence of various factors on the growth of *Staph. aureus* in bacon, an experiment was set up in which two types of bacon were each inoculated with large and small numbers of staphylococci: samples of each were then stored at either 5° C. for up to 42 days or 15° C. for up to 21 days.

Staphylococcal culture

The culture no. 69/7015, phage type 85, had been isolated from ham and implicated as a source of a small (family) food-poisoning outbreak. It had been shown to produce enterotoxin A. After passage through 2–3 transfers in nutrient broth (NB) (each passage consisted of incubation for 24 hr. at 37° C.), and before inoculation into the bacon, the culture was serially diluted in quarter-strength Ringer's solution and plated on the tellurite–glycine–egg yolk medium (EGPTA) of Baird-Parker (1962). The bacterial population per ml. was calculated after incubation at 37° C. for 48 hr.

Bacon

A boned-out Wiltshire middle was cured and sliced in the factory and a second middle was cured according to the 'hygienic cure' procedure (Dempster, 1972). Before slicing, the second middle was dipped in a 10 gal. milk churn of boiling brine for 15 sec. with the object of reducing surface bacterial numbers. Both middles were sliced and sufficient slices then transported to the laboratory without delay and each divided into equal sub-lots. With aseptic care, individual slices were laid out on sheets of grease-proof paper and inoculated with a suspension of the test organism. One half of each subsample of bacon was seeded with a 'high density' inoculum and the second half with a 'low density' inoculum as follows: 0·5 ml. NB. culture was added as drops (0·1 ml. per drop) on to each slice using a graduated pipette. The drops were placed along the surface of the muscle. The number of staphylococci in the high-density inoculum was 168×10^6 per ml. and the number in the low-density inoculum was $43{\cdot}5 \times 10^3$ per ml.; this gave approximately 10^6 organisms/g. and 10^3 organisms/g. based on the weight of bacon in each pack. Two inoculated slices were then sandwiched and placed in a 'Metathene' (X320) pouch and vacuum-drawn. Sufficient material was treated to provide for the analysis of triplicate packs at each storage interval. Two sub-lots were stored at 5° C. for 6 weeks and sampled at weekly intervals. Two further sub-lots were stored at 15° C. for 3 weeks and sampled twice weekly. Uninoculated control slices of Wiltshire and 'hygienic-cured' bacon were similarly packaged and stored at the two temperatures.

Bacteriological analyses of bacon

At sampling the packs were opened aseptically and the entire contents macerated with scissers. Ten gram amounts from each pack were then transferred to 3 × 20 cm hard-glass Pyrex boiling tubes and 40 ml. of sterile water added. The mixture was blended for 1 min. on a 'Polytron' homogenizer and 0·1 ml. serial dilutions in Ringer–peptone solution added to poured plates of EGPTA and Oxoid Plate Count Agar (PCA) + 3 % of added salt. Plates were counted after 24 hr. at 37° C. (EGPTA) and 3 days at 25° C. (PCA).

Chemical analyses of bacon

The lean from the remaining macerate was minced and duplicate samples (5 g.) weighed into porcelain dishes. The samples were dried for 18 hr. at 100° C. and

Table 1. *Total colony count, coagulase-positive staphylococcal counts, salt, nitrite and pH of uninoculated bacon*

Bacon	Total colonies* on PCA +3% NaCl	Coagulase-positive staphylococci* on EGPTA	NaCl (%, w/v)	Nitrite (μg./g.)	pH
Wiltshire	5·03	< 1·69	4·14	51	6·2
'Hygienic'	3·94	< 1·69	2·71	318	5·85

* Log_{10} colonies/g.

ashed for a further 18 hr. at 550° C. Chloride was determined on the ash by Volhard's method (Vogel, 1948). Nitrite was estimated by the method of Follet and Ratcliffe (1963) using 10 g. of minced lean. The pH values of the homogenates were measured with a portable meter (Radiometer, Copenhagen) standardized against a pH 6·50 phthalate buffer.

RESULTS

The bacterial population and chemical composition of samples of the two bacons before inoculation with the test organism are shown in Table 1.

Storage at 5° C.

The survival of the test organism at 5° C. in both the Wiltshire and hygienically cured vacuum-packaged bacon stored for 6 weeks is presented in Fig. 1. Numbers increased marginally in the 'high density' Wiltshire bacon (Fig. 1*a*) up to 14 days ($10^{6 \cdot 265}$ to $10^{6 \cdot 44}$) and in the hygienic sweet-cured bacon (Fig. 1*c*) up to 7 days ($10^{6 \cdot 18}$ to $10^{6 \cdot 46}$). Thereafter numbers decreased. However, even after 6 weeks, 94·7% and 87·8% of the initial staphylococcal numbers were still viable in the Wiltshire and 'hygienic' bacons respectively. The total viable count (inoculated samples) increased progressively, reaching maximum numbers in 35 days (Wiltshire) and 42 days (hygienic cure). Despite the initial rise in growth the numbers of staphylococci did not increase in either bacon.

In both 'low density' bacons (Figs. 1*b*, 1*d*) staphylococci decreased throughout storage; only 65·0% and 62·0% of the original inoculum was still viable in the Wiltshire and 'hygienic' bacons respectively at the end of storage. The total viable count increased to reach maximum numbers in 35 days in both types of bacon. No coagulase-positive staphylococci were isolated from uninoculated control packs stored at 5 °C.

Storage at 15° C.

The fate of *Staph. aureus* on bacon stored for 3 weeks at 15° C. is shown in Fig. 2. Numbers increased in the 'high density' Wiltshire (Fig. 2*a*) up to 11 days and in the 'high density' 'hygienic' bacon (Fig. 2*c*) up to 17 days. Staphylococci did not die out; the final count was about equal to the initial count on the Wiltshire and exceeded the initial count (112·0%) on the 'hygienic' bacon. The total viable count reach maximum numbers in 7 days on the Wiltshire and in 17 days on the

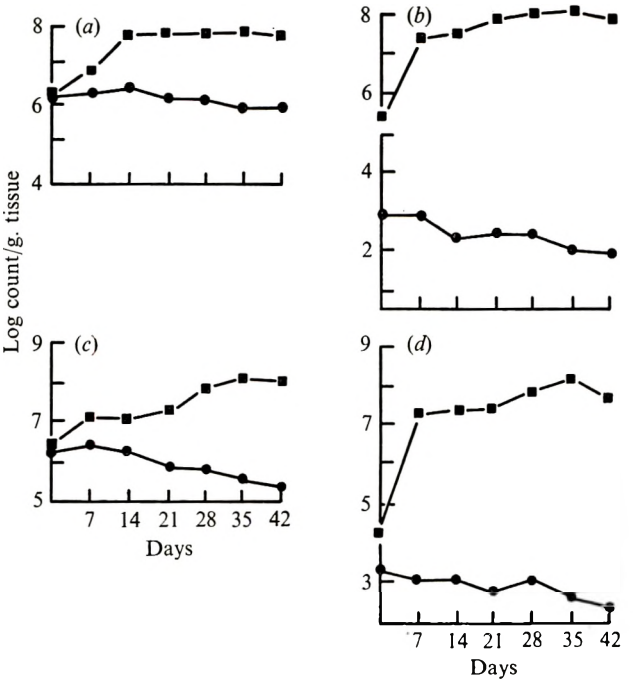

Fig. 1. Changes occurring in Wiltshire (*a*, *b*) and hygienic (*c*, *d*) sweet-cured vacuum packaged bacon at 5° C., inoculated with high (*a*, *c*) and low (*b*, *d*) levels of *Staphylococcus aureus*. Squares, total viable count on PCA + 3 % NaCl; circles, Staphylococcus count on ETGPA.

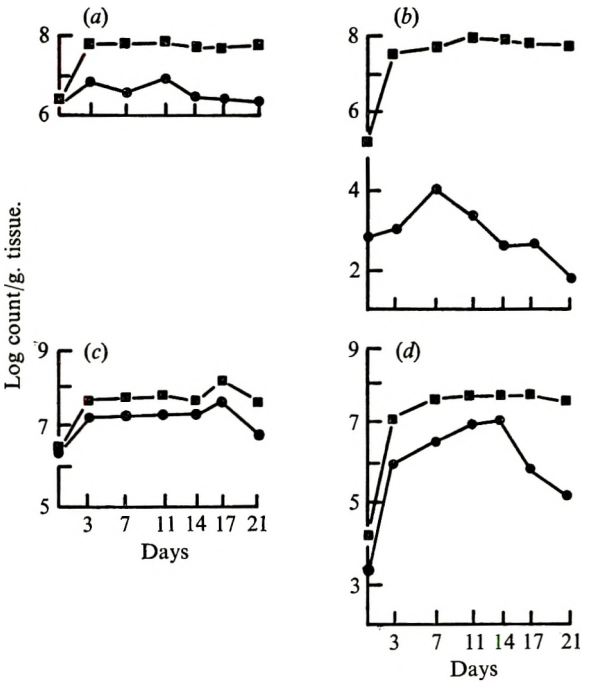

Fig. 2. Changes occurring in Wiltshire (*a*, *b*) and hygienic (*c*, *d*) sweet-cured vacuum-packaged bacon at 15° C., inoculated with high (*a*, *c*) and low (*b*, *d*) levels of *Staphylococcus aureus*. Squares, total viable count on PCA + 3 % NaCl; circles, 'Staphylococcus' count on ETGPA.

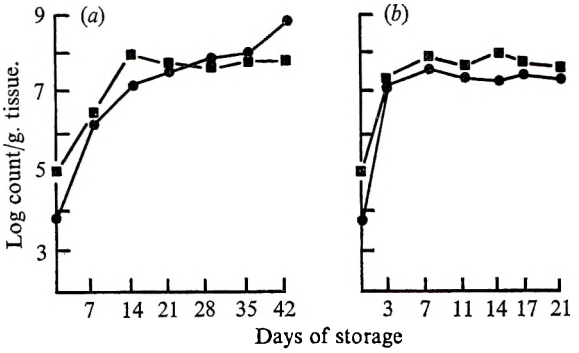

Fig. 3. Changes in total count (PCA + 3 % NaCl) in vacuum-packaged bacon (uninoculated controls) stored at (*a*) 5° C. and (*b*) 15° C. Squares, Wiltshire; circles, hygienic, cured bacon.

Table 2. *Composite pH values of vacuum-packaged bacon held at 5° C.*

Days of storage	Wiltshire Bacon inoculated with: *c.* 10^6/g	*c.* 10^3/g	Control	'Hygienic' Bacon inoculated with: *c.* 10^6/g	*c.* 10^3/g	Control
0	6·20	6·20	6·20	5·85	5·85	5·85
7	5·68	5·63	5·60	6·01	5·90	5·80
14	6·05	5·87	5·90	6·23	6·13	5·90
21	6·01	6·00	5·50	6·06	6·00	5·40
28	5·51	5·55	5·50	5·65	5·68	5·50
35	5·56	5·58	5·40	5·65	5·73	5·55
42	5·71	5·71	5·50	5·85	5·85	5·70

Table 3. *Composite pH values of vacuum-packaged bacon held at 15° C.*

Days of storage	Wiltshire bacon inoculated with: *c.* 10^6/g	*c.* 10^3/g	Control	'Hygienic' Bacon inoculated with: *c.* 10^6/g	*c.* 10^3/g	Control
0	6·20	6·20	6·20	5·85	5·85	5·85
3	5·86	5·76	5·60	5·68	5·93	5·70
7	5·45	5·80	5·45	5·51	5·56	5·65
11	5·63	5·63	5·30	5·50	5·66	5·30
14	5·78	5·91	5·85	5·95	5·80	5·75
17	5·56	5·63	5·30	5·72	5·70	5·60
21	5·65	5·53	5·50	5·45	5·60	5·20

'hygienic' bacon. In the 'low density' bacons (Figs. 2*b*, *d*) staphylococci increased to maximum numbers in 7 days on the Wiltshire and in 14 days on the 'hygienic' bacon. Thereafter numbers decreased to less than the initial level on the Wiltshire (< 58·0 %) but in excess of the initial inoculum on the 'hygienic' bacon. Total viable numbers increased considerably during the first 7 days on both bacons to maxima in 11 days on the Wiltshire and 17 days on the 'hygienic' bacon. Thereafter numbers decreased slightly. Again no coagulase-positive staphylococci were isolated from control packs stored at 15° C.

The total bacterial count in uninoculated samples of both bacons held at 5° and

15° C. is presented in Fig. 3. Total numbers increased to maxima in 35 days at 5° C. and 14 days at 15° C. (Wiltshire) and in 42 days at 5° C. and 7 days at 15° C. ('hygienic' bacon).

The pH (mean of triplicate readings) of the bacon homogenates is shown in Tables 2 and 3. The pH decreased significantly from 6·20 to values between 5·71 and 5·53 in the Wiltshire bacon at both 5° and 15° C. and inoculated with high and low densities of staphylococci. There was little change, however, in the pH of the 'hygienic' bacon at either temperature or at either inoculum density.

DISCUSSION

In the experiments reported here, the effect of concentration of curing ingredients as well as the method of cure, storage temperature and the numbers of staphylococci artificially introduced into vacuum packaged bacon were studied. A coagulase-positive staphylococcus grew slowly in packaged bacon at 5° C. but only when the bacon was inoculated with high numbers. The fact that the numbers decreased over the experimental periods in both types of bacon after inoculation with small numbers suggests that the inoculum density *per se* exercises a significant effect on the ability of staphylococci to grow at low temperatures irrespective of the numbers or nature of the competing microflora. Thus in bacon inoculated with a 'high density' of staphylococci, 94·0 % and 87·0 % of the original numbers were still viable after 6 weeks at 5° C. in the Wiltshire and 'hygienic' bacon respectively. In the 'low density' bacons at 5° C. only 65·0 % (Wiltshire) and 62·0 % ('hygienic' bacon) of the initial inocula were still viable. This result agrees with the findings of Peterson, Black & Gunderson (1962), who observed a definite respressive effect on the growth of staphylococci when a mixture of saprophytic and psychrophilic bacteria was present. This effect was more pronounced as the staphylococcal portion of the population decreased. Obviously this is an example of Ingram's (1962) population-pressure hypothesis. Similar observations were made by Di Giacinto & Frazier (1966), who determined the inhibitory effect of coliform bacteria and *Proteus* spp. on the growth of staphylococci and showed that the time necessary for 2×10^4 staphylococci to reach 5×10^6 per ml. varied with the species of inhibiting ('effector') organism, the original ratio of effector organism to staphylococci, and the incubation temperature.

In the experiments described here, the test organism multiplied in both types of bacon held for 3 weeks at 15° C. Only in the 'low density' Wiltshire samples was the final count less than the original (< 58·0 %); in the 'high density' Wiltshire bacon 100·0 % of the initial inoculum was still viable. In both the 'high' and 'low' density 'hygienic-cured' bacon, the final staphylococcal count was 112·0 % and 115·0 % of the initial count respectively. Thus inhibition of staphylococci by other micro-organisms is affected by temperature; being less as the temperature rises. This has already been established, especially when bacon is stored at temperatures exceeding about 25° C. (Eddy & Ingram, 1962; Ingram, 1960). It was suggested by Eddy & Ingram (1962) that the principle obstacle to the multiplication of staphylococci is the preponderance of the normal microflora and they

posed the question, 'why does this flora inhibit staphylococci?'. Sufficient evidence is available to show that certain genera, species and groups of bacteria exercise an inhibitory effect on staphylococci. Thus Graves & Frazier (1963) showed that *Lactobacillus* spp., *Leuconostoc.* spp, and *Streptococcus* spp. were inhibitory. Presumably the inhibition in this case was due to a lowering of pH. Acid pH is inimical to the growth of staphylococci as was shown by Bardsley & Taylor (1960). In bacon treated with lactic acid (pH 5·42) staphylococci failed to grow in 15 days while in control samples (pH 5·76), the test organism multiplied significantly. In fact these workers suggested that pork of low pH ($< 6{\cdot}0$) should be chosen for vacuum-packaged bacon as presenting conditions least favourable to growth of staphylococci. The results of the present experiment suggest that in no instance was the material acid enough to be solely responsible for staphylococci failing to grow.

Graves & Frazier (1963) also showed that certain other organisms, e.g. *Bacillus cereus*, *Proteus vulgaris* and *Escherichia coli*, produced an inhibitory substance specific for *Staphylococcus aureus*. Faecal streptococci and meat lactobacilli were shown to be inhibitory by Oberhofer & Frazier (1961), the inhibition varying with temperature and the test medium.

Staphylococci are extremely tolerant of salt, and many of the selective media recommended for their cultivation contain high concentrations of sodium chloride, e.g. the mannitol–salt agar of Chapman (1945) and staphylococcus medium 110 (Chapman, 1946). It is therefore clear that *Staph. aureus* and associated types (coagulase-negative staphylococci and micrococci) can flourish in salt concentrations considerably in excess of those present in bacon at any stage of the curing process. The salt content of the Wiltshire (4·14 %) or of the 'hygienic' bacon (2·71 %) would in no way restrict the growth of staphylococci.

The effects of curing ingredients and pH on the survival of staphylococci were studied by Lechowich, Evans & Niven (1956). They showed that in a basal medium containing salt, nitrate and nitrite, anaerobic tolerance to nitrite was in the range 100–200 parts per million (p.p.m.) and growth was prevented at pH 5·60. The nitrite content and pH of the bacon in the present study would not therefore be inhibitory in this sense. Likewise the work of Castellani & Niven (1955) showed that *Staph. aureus* could tolerate 800 p.p.m. NO_2 under anaerobic conditions at pH 6·55. Tarr (1941, 1942) studied the effect of NO_2 at 200 p.p.m. on a wide range of organisms and found the bacteriostatic effect increased markedly with decrease in the pH of the medium. At pH 5·60, 200 p.p.m. NO_2 gave partial inhibition of *Staph. aureus*.

Staphylococci are widely distributed in nature; the very high frequency with which they occur in the nasal passage of humans is shown by the work of several investigators. Thus Miles, Williams & Clayton-Cooper (1944) obtained incidences of between 19·0 and 65·0 %. More recently, Ravenholt, Eelkema, Mulhern & Watkins (1961) found that 32·0 % of 318 workers in 15 meat-handling plants in the U.S.A. carried coagulase-positive staphylococci on their skin. Obviously then, a considerable proportion of all people handling food are liable to infect it with staphylococci either directly or indirectly. The danger increases in cases of respira-

tory infection, causing coughing and sneezing. In addition, food handlers with infected cuts or skin infections like boils and pimples will heavily contaminate food. It is not unreasonable to assume that vacuum packaging with its attendant handling predisposes bacon to potential contamination with staphylococci. Hansen & Riemann (1962) have suggested that full security against staphylococci growing in vacuum packs would be realized if prophylactic and compositional control of the product were implemented. By prophylactic control they meant bacteriological control, judicious selection of personnel and handling of the product under aseptic conditions. By composition control they included the effects of concentration of curing ingredients and water activity. Vacuum-packaged bacon invites abuse by storage at ambient temperature (Kitchell & Ingram, 1963). This was demonstrated forcibly by Thatcher, Robinson & Erdman(1962), who showed that staphylococci reached 10^8 to 10^9 per g. in bacon stored anaerobically at 37° C. and that toxin was produced even though the product was organoleptically acceptable.

Cured meats have a bacterial flora that is quite different from that of fresh meats. This is due to the selective bacteriostatic activity of the curing salts. The major selective action is due to sodium chloride. However, one of the most important groups of bacteria that is not inhibited by any palatable salt concentration is the staphylococcus group which includes food-poisoning types. Thus staphylococci grow on cured products, being halotolerant, whereas salmonellas and clostridia do not. The tolerance of staphylococci to nitrite is much lower under anaerobic conditions (Castellani & Niven, 1955) and it seems probable that at the low oxygen tension and low pH likely in a vacuum pack, sufficient nitrite might often be present to prevent their growth although this was not observed in the present investigation.

Although the total bacterial count of the 'hygienic' bacon was about one-tenth that of the Wiltshire bacon initially, total numbers reached about the same level in both bacons during storage and yet the staphylococcus was repressed in the Wiltshire but not in the 'hygienic' bacon. Thus suggests that it may not be simply a question of numbers as suggested by Eddy & Ingram (1962), but rather types of organisms which exercise the inhibitory effect.

From the above observations it appears that coagulase-positive staphylococci can survive in vacuum-packaged bacon at temperatures much lower than the optimum (37° C.) for this organism. However, to cause food poisoning, staphylococci must multiply sufficiently to produce toxin and little is known about the optimum conditions for toxin production (Wilson & Miles, 1964). Thatcher *et al.* (1962) showed that only a small amount of enterotoxin was produced in vacuum-packaged bacon at 37° C. even though the count of staphylococci had reached 10^9 to 10^{10} per g. McCoy & Faber (1966) studied the influence of food micro-organisms on staphylococcal growth and toxin production in meat and concluded that staphylococcal numbers cannot be used as an index of toxin formation. Therefore the aim should be to prevent contamination of food in the first instance. However, prevention and control are fraught with difficulty because of the ubiquitous nature of the organism, by the fact that the infected food is not altered in appearance, taste or smell, and by the heat-resistant nature of the toxin. Prevention must

depend greatly upon such factors as general hygiene, personal cleanliness and the protection of food during preparation and storage.

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A particle detector for use in ventilation engineering

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SUMMARY

An electronic particle counting device is described, based on a constant-temperature hot-wire anemometer probe. The device has found application in tracing the motions of droplets and measuring their concentration within ventilated areas when subjected to different types of ventilation. In this way the effectiveness of various systems may be determined.

INTRODUCTION

The need to detect and count the numbers of tracer aerosol droplets within a room ventilated by two different methods led to the development of the particle detector described in this paper and is an extension of the apparatus described by Clark, Cox & Lewis (1971).

The basis of the device is a constant-temperature hot-wire anemometer on to which particles from an air stream land. Goldschmidt (1965) showed that by suitable electronic filters the signal associated with the cooling of the wire due to a particle landing on it could be discriminated from the cooling effect due to varying air velocity past the wire.

The present device employs a hot wire anemometer probe enclosed within a nozzle. The electronic circuits are battery-powered and the apparatus is portable when used with a battery-operated electronic counter. Fig. 1 shows a block diagram of the component parts of the detector, and Plate 1 shows the complete apparatus.

THE SAMPLER

Principle of operation

When a particle lands on the heated wire the wire rapidly loses heat to the particle. The voltage output from the wire that is associated with this heat loss is distinguishable from that due to air velocity fluctuations past the wire. The waveforms due to the particle impacts have a very steep slope which reach a sharp peak and then die away quickly. The peak voltage associated with the impact of a 30 μm. diameter particle is about 300 mV. When the particle hits the wire there may also be a 'strain gauge' effect which may change the resistivity of the wire. However, this is assumed to be a small effect since particle impacts have also been detected using a hot film probe where this effect is negligible.

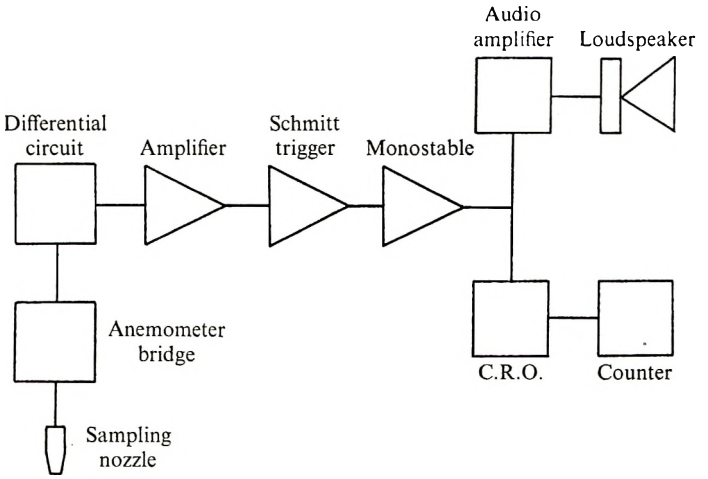

Fig. 1. Block diagram of the sampling apparatus.

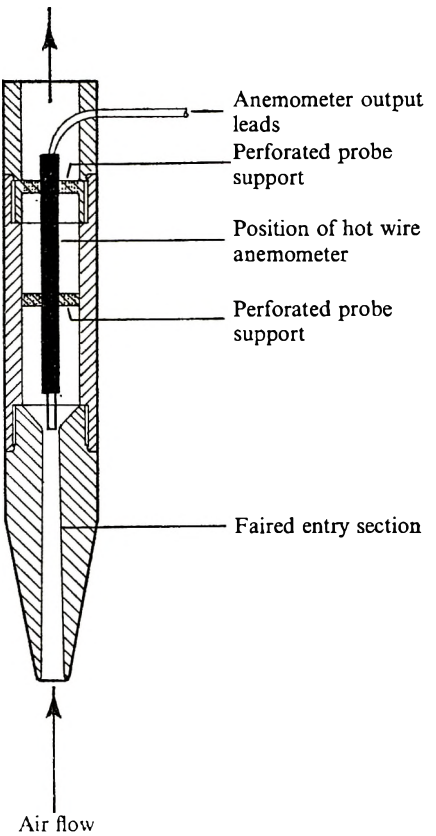

Fig. 2. Section through the sampling nozzle.

Goldschmidt also showed that the sensitivity of the wire to particle impacts was inversely proportional to the wire diameter and length, and proportional to the temperature coefficient of resistivity of the wire.

Therefore, for large overheat ratios, large air velocities past the wire and low values of wire resistivity, the chances of discriminating particle impacts will increase.

Description of the sampling nozzle

The hot-wire anemometer probe consists of a moulded epoxy-resin body in which are embedded two nichrome wire prongs of 0·025 in diameter. The distance between the prongs is 2 mm. and across the ends of the prongs a 5 μm. diameter tungsten wire is welded. The hot-wire probe is located in a sampling nozzle and a drawing to show the section through the nozzle is shown in Fig. 2.

The nozzle body is made in three sections from aluminium and the probe itself is held centrally in the nozzle body by two probe supports. These supports are perforated to allow the air to pass through the nozzle. The 5 μm. diameter wire on the end of the probe is positioned just at the end of the faired entry section of the nozzle. Flexible plastic tubing is attached to the end of the nozzle and connected to a suction pump. Plate 1 shows the complete assembly together with a battery-operated pump (Micronair Type 3900-10). The output from the hot-wire probe is fed to the anemometer bridge circuit. This is either a DISA 55D05 battery-operated bridge or a DISA 55D01 model which allows the wire to be operated at a maximum overheat ratio of 1·8.

Circuit description

Fig. 3 shows the complete circuit diagram for the sampler. The output from the anemometer bridge is differentiated and then fed to an amplifier. This amplifier output passes to a Schmitt trigger and then to a monostable circuit which provides an output to an electronic counter and a storage oscilloscope. The monostable output is also fed into an audio amplifier coupled to a loudspeaker. Plate 2(*a*) shows separately the output signals from the anemometer probe. The top trace shows the 'spikes' associated with a particle impact on the wire, and on the bottom trace is shown the repetitive signal due to the velocity fluctuations at the wire produced by the reciprocating air-pump. These two signals are superimposed at the anemometer output and the composite signal is fed to the differential circuit and first amplifier, where the signal from the air pump is attenuated to a greater extent than the 'spikes' of the particle impacts.

Plate 2(*b*, c) illustrates the action of this part of the circuit when sine and square-wave signals are injected into the sampler circuit. These sine and square waves are analogous to the pump and particle signals respectively. Plate 2(*b*) shows these input sine and square waves which are of similar magnitude, and Plate 2(*c*) shows, on the same vertical scale, the resultant signal just after the differential circuit and amplifier. It is seen that the differentiation of the square wave produces a signal with no amplitude attenuation whereas the sine wave undergoes an attenuation of about two-thirds the input voltage. It is in this way that the superimposed particle

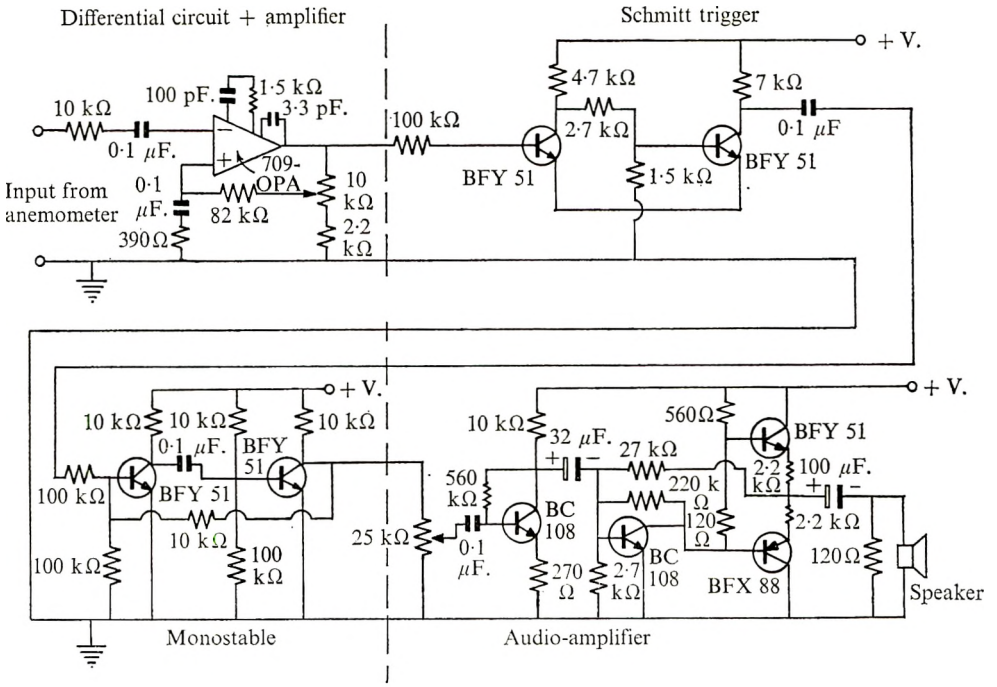

Fig. 3. Circuit diagram of particle sampler.

and pump signals are discriminated with the result that the effect of the particles is enhanced and that of the pump minimized.

These voltage-pulse differences are next fed to the Schmitt trigger. This trigger is adjusted to respond only to pulses of greater amplitude than those associated with the velocity fluctuations. The pulses that pass this trigger, due only to the particle impacts, are fed into the monostable circuit. This produces a constant amplitude voltage output pulse for all amplitudes of the Schmitt trigger output. The electronic counter and storage oscilloscope respond to the monostable output and indicate the number of particle impacts on the wire.

By feeding the monostable circuit into the audio amplifier and loudspeaker a signal is also heard for each particle impact.

Efficiency of the sampler

Experience has shown that the sampler is more sensitive to liquid aerosol droplets than to solid particles. Consequently for tracing the motion of particles in ventilation systems, liquid aerosol droplets up to 30 μm. diameter have been used as the tracer particles. The efficiency of the sampling wire is the ratio of the number of particles that land on the wire to the number of particles, the centres of which would have passed through it if they had moved all the time in straight lines. For purely inertial deposition this efficiency only depends on the particle stop distance. The ratio of this stop distance to a characteristic dimension of the system (i.e. the wire radius) is the Stokes number.

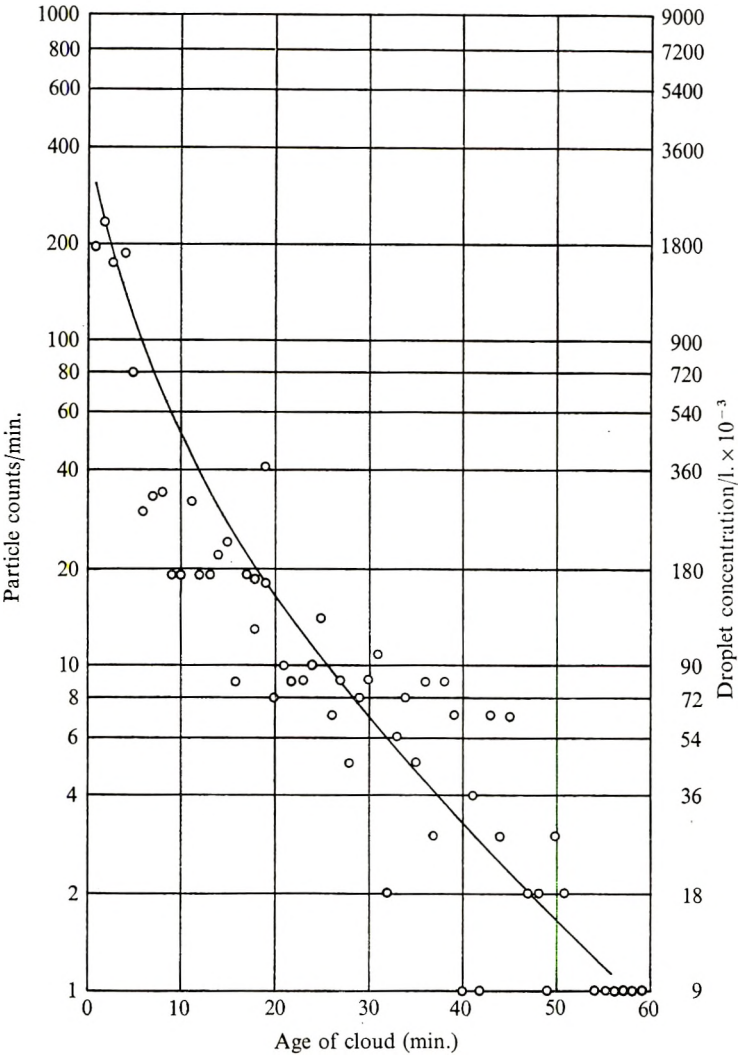

Fig. 4. Decay of a cloud of aerosol droplets in a room during 1 hr.

The collection efficiency for particles landing on a cylinder has been calculated (Fuchs, 1964) and in the case of a 30 μm. diameter particle hitting a 5 μm. diameter wire this efficiency is about 98 %.

The air pump used with this sampler produces an air flow of 1·4 l./min. past the heated wire and the mean air velocity over the wire at the end of the faired entry is about 180 cm./sec.

Use of the sampler

This sampler finds application in air conditioning or heating systems where tracer particles can be introduced into the air stream and their concentrations subsequently sampled. Two illustrations of its use are given here. Fig. 4 shows the decay of a cloud of aerosol droplets due to sedimentation in a room with relatively little air movement. Upwards of 40 min. was required before the particle count returned to that before the aerosol was injected.

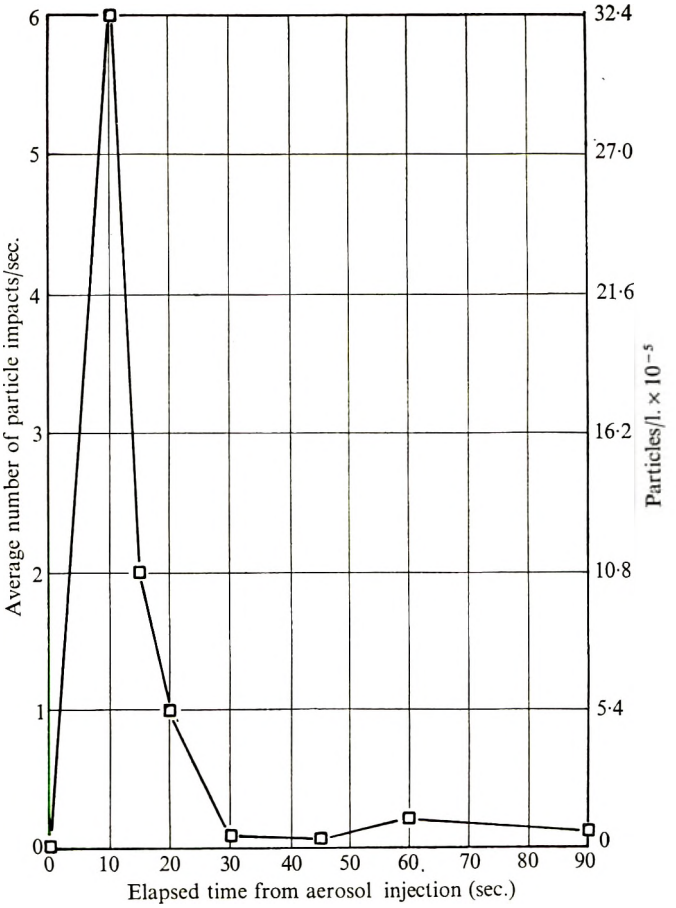

Fig. 5. Tracer droplet concentration levels in the centre of a chamber with a horizontal air flow of 25 cm/sec.

Fig. 5 shows the way in which particle concentration within a horizontal linear (laminar) flow room changed after the injection of tracer particles upstream of the sampling nozzle. The particle count rose steadily for the first 20 sec. and then the rate of counting decreased rapidly as the particles were blown through the room and into the filters.

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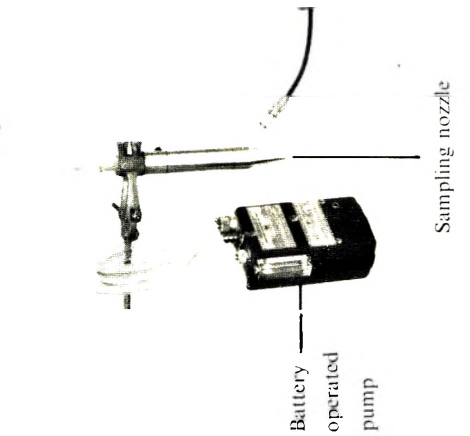

R. P. CLARK

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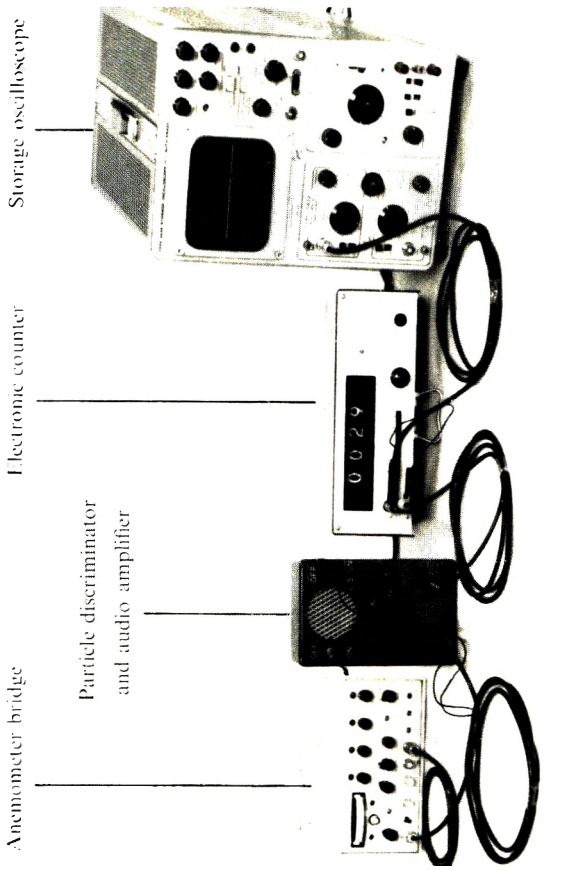
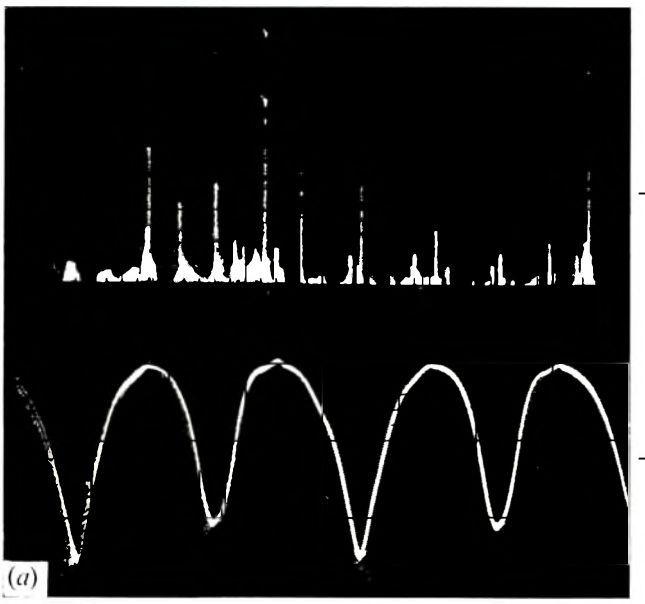
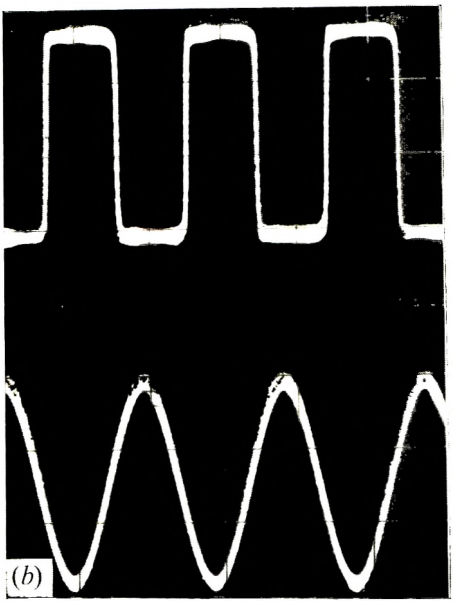
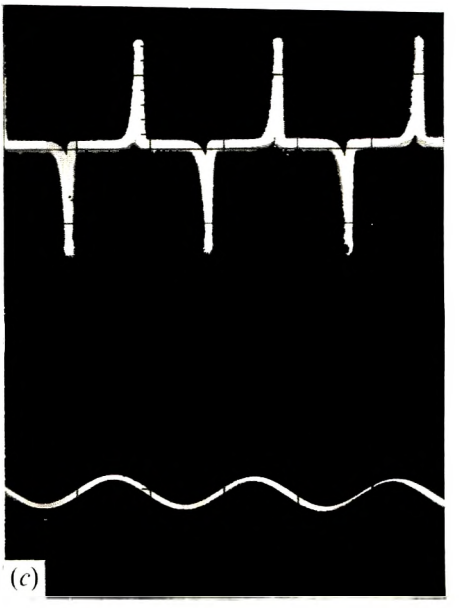

Storage oscilloscope
Electronic counter
Particle discriminator and audio amplifier
Anemometer bridge


Anemometer output signals
'Spikes' due to particle impacts
Signal due to the air velocity past the hot-wire probe
(a)


(b)


(c)

EXPLANATION OF PLATES

Plate 1

The complete particle detector.

Plate 2

(*a*) Anemometer output signal. Upper line: 'spikes' due to particle impacts. Lower line: signal due to air velocity past the hot-wire probe.

(*b*, *c*) The effect of the differentiator and first amplifier on both square and sine waves. (*b*) Injected square and sine waves of equal magnitude. (*c*) the differentiated signal. The square waves produce sharp peaks of similar magnitude to the injected signal.

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Thermal comfort zones obtained by two alternative methods: a note

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In correspondence arising out of our recent paper (Wyon, Andersen & Lundqvist, 1972), interest has been expressed in the relative widths of the comfort zones obtained by using the Bedford 7-point scale and our proposed dial-vote scale. It was possible to make a direct comparison on our original data, using votes registered simultaneously on the two scales at the end of each hour of exposure. Air temperature rose only slowly towards the end of each hour, and the comparison is probably valid also for steady-state conditions. The proportions voting 'too hot' (Bedford categories 6, 7, dial vote $>66{\cdot}6\%$) and 'too cold' (Bedford categories 1, 2, dial vote $<33{\cdot}3\%$) were derived and probit analysis was performed (Finney, 1947). Only responses obtained from the 36 female subjects are considered, for very few males were too cold in the temperature range 23–29° C. The results are shown in Fig. 1, using the same presentation as in fig. 7 of the original paper. No statistically significant differences could be shown between the pairs of regression lines, either in terms of their slopes or median values. However, the tendency is clearly for the dial-vote method to yield if anything a rather narrower and lower comfort distribution, and hence a narrower zone of comfort, however defined. This in spite of a marked 'comfort zone' occupying as much as one-third full scale. As pointed out in our original paper, the comfort zone yielded by either scale should be regarded as a zone of tolerance and not as a zone of ideal comfort.

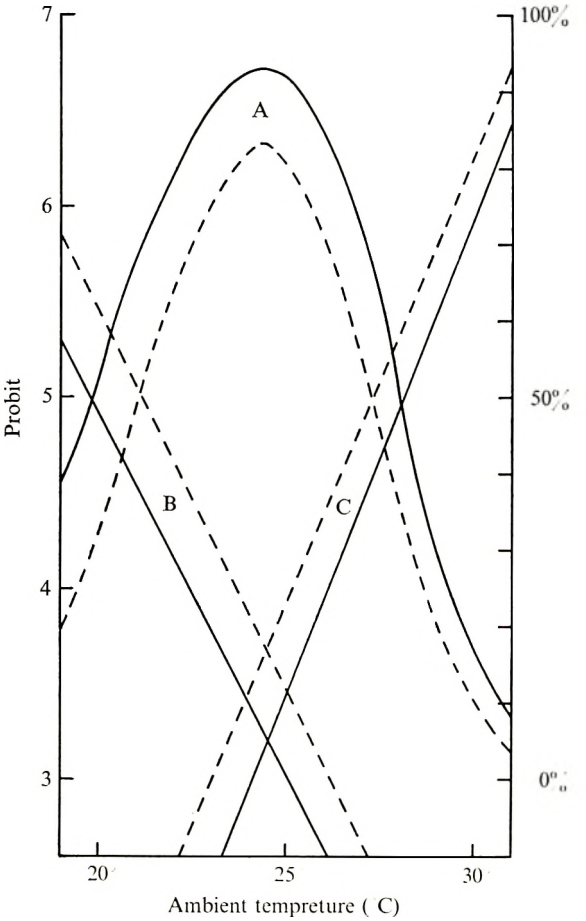

Fig. 1. Proportion of 17-year-old female subjects, wearing standard clothing of insulation value 0·7 clo, who were comfortable, uncomfortably cold and uncomfortably hot at various temperatures. The unbroken lines depict responses obtained using the Bedford 7-point scale, and the broken lines depict those obtained simultaneously using the dial vote scale. Curves A show the percentage comfortable; right-hand vertical scale. Lines B and C are the probit regression lines, left-hand vertical scale, for the proportions feeling too cold and too hot respectively.

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The effect of saline on the eye irritation caused by swimming-pool water

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SUMMARY

In laboratory experiments the acute eye irritation produced by exposure to tap water was not significantly increased when chlorine compounds were added to the water at concentrations of 1 mg./l. The greatest irritation was produced by 2 mg. Cl_2/l. as NH_2Cl. The addition of NaCl at concentrations above about 0·5% abolished the irritant effect of tap water, and prevented irritation even when 1 mg. Cl_2/l. was present.

In a field experiment involving two swimming baths, one with fresh and the other with saline water (0·5% NaCl), eye irritation in the saline bath was significantly lower than in the freshwater bath only when the swimming time did not exceed 30 min.

INTRODUCTION

Eye irritation after swimming in chlorinated swimming-pool water is a well-known phenomenon. Extensive discomfort may occur especially among swimmers who train for several hours daily. Theoretically, the agents responsible for the eye irritation are the water temperature, pH, the chlorine concentration, the concentration of organic matter especially nitrogen compounds and the total and even the relative concentrations of various inorganic salts, including residues of the aluminium salts used in chemical purification. In addition, swimmers may have an individual sensitivity to any factor.

Despite the importance of the problem only a few papers have been published on the significance of the various agents mentioned above. Mood, Clarke & Gelperin (1951) reviewed earlier investigations and reported their own results. In the first of these, available chlorine over 0·7 mg Cl_2/l. was found to result in eye irritation for swimmers; in the second report no eye irritation attributable to chlorine was noted at concentrations up to and including 1 mg. Cl_2/l.; and in the third report free available chlorine of 2·0 mg Cl_2/l. was stated not to cause skin or eye irritation.

In Mood's own investigation subjects were asked to swim free-style for approximately 11 min. in a swimming-pool where the water was treated by means of aluminium sulphate coagulation and pressure filtration through sand, a soda ash additive and sterilization with chlorine gas. Before and after the swim the eye irritation was measured both objectively and subjectively. By increasing the total

available chlorine from 0·05 to 0·5 mg. Cl_2/l. (of which 0·4 was free available chlorine) an increased frequency of complaints from subjects on eye irritation was obtained. When the pH was lowered from 8·0 to 7·0, an even higher frequency of eye irritation was found. The objective eye examinations showed no correlation with chlorine concentration or pH.

Schein, Tammelin & Zetterström (1951) compared three pools with different water-treatment processes. In one the water was treated by means of $AlSO_4$ coagulation, rapid filtration through sand, alkalization to pH 7·0 and chlorination to 0·25 mg. Cl_2/l. combined available chlorine. In the second the treatment was by filtration through alkaline $MgCO_3.CaCO_3$ to pH 9·7 and break-point chlorination up to 2·3 mg. Cl_2/l. free available chlorine. The third process involved filtration through sand, soda ash additive to pH 8·8 and break-point chlorination to 2·3 parts per million (p.p.m.) free available chlorine. School-children were used as subjects and asked to swim for 10 min., after which their eyes were examined. Subjective reactions were not recorded. The frequency of reddening of the conjunctiva was found to be about equal among the swimmers in the three different pools while the frequency of mild swelling and slight bleeding of the conjunctiva was significantly lower in the pool where the water was treated by the second process described above.

The purpose of the present investigations was to study the acute effects of pH, chlorine concentration, various chlorine compounds, and the salt concentration for the eye irritating properties of water under standardized laboratory conditions. In addition, a limited field study was performed to test the hypothesis derived from the laboratory experiments.

LABORATORY STUDY

Materials and methods

In the experiments two different water solutions were held against either eye for 1·5 min. in eyewash cups. The subjective feeling of irritation was determined and the difference between the two eyes was recorded. Various solutions were applied at random to the right and left eyes to avoid any systematic error based on a difference in sensitivity between the eyes. The studies were made on 7–10 subjects for each type of water tested. Hypochlorite, monochloramine and chloramine B (sodium benzenesulphochloramide) were studied in different concentrations with varying concentrations of NaCl added. In all experiments the pH was kept at 8·3, except in one experiment when it was 7 and 9 in the two solutions to be compared.

Chlorine demand-free water was produced by adding sodium hypochlorite to tap water to 4 mg. Cl_2/l., and leaving it for 3 days to oxidize all chlorine-binding substances. The main part of the active chlorine was then removed with a saturated sodium sulphite solution and the last traces by boiling the water for approximately 20 min. De-chlorinated and chlorine demand-free tap-water of this kind was used in all the experiments. The pH was regulated with NaOH or HCl. The temperature of the water was about 22° C. in all experiments. The saline content was regulated using sodium chloride (P.A. quality). The chloride ion concentration in the tap-

Table 1. *Experimental design for testing of water with different composition*

Basic test solution	Variables		
Water	pH	Chlorine concentration	Chlorine compounds
Water + 0·7 % NaCl	pH	Chlorine concentration	Chlorine compounds
Water	NaCl concentration	NaCl concentration in 2 mg. Cl_2/l. HOCl	NaCl concentration chlorine concentration

Table 2. *Reaction to various types of water*

Variable	Reaction
NaCl concentration	No irritation at $\geq$ 0·5 %
NaCl concentration in 2 mg. Cl_2/l. HOCl	No irritation at $\geq$ 0·7 %
pH variation with or without chlorine	Irritation not related to pH except perhaps with 0·7 mg. HOCl
Chlorine concentration in	
(*a*) Water	Slight additional irritation in certain subjects at 1·0 mg Cl_2/l. Strong additional irritation at 2·0 mg. Cl_2/l. as NH_2Cl
(*b*) Water + 0·7 % NaCl	Almost no irritation at 1·0 mg Cl_2/l. Irritation at 2·0 mg Cl_2/l. as NH_2Cl
Chlorine compounds	NH_2Cl more irritating than HOCl and Chloramine B

water was 10 mg./l., which equals about 0·0014 % NaCl. Free available chlorine was obtained by adding commercial sodium hypochlorite solution. The inorganic chloramine solutions were produced by mixing 1 mg. NH_4^+/l. as NH_4Cl. with the corresponding prediluted hypochlorite concentrations. The organic chloramine (chloramine B) was obtained from a solution of a commercial product. The concentrations of chlorine compounds were expressed as measured available residual chlorine in mg. Cl_2/l.

The test solutions for the eyes were varied according to the experimental design model shown in Table 1.

Results

Tap-water by itself was found to be unpleasant and irritating when tested experimentally. The results of testing various types of water according to Table 1 are summarized in Table 2. The results show that the acute eye irritation produced by tap-water was not significantly increased when hypochlorite monochloramine, or chloramine B was added at 1 mg. Cl_2/l.: 2 mg. Cl_2/l as NH_2Cl caused very strong irritation, but 2 mg. Cl_2/l. as HOCl did not significantly increase the irritation produced by water.

When NaCl was added at concentrations above about 0·5 % the irritating proper-

Table 3. *Chemical characteristics of the water at the two baths*

	Bath 1 (tap-water)	Bath 2 (saline water)
Permanganate no. ($KMnO_4$ mg./l.)	10	13
NH_4 (mg./l.)	0·6	0·4
NO_3-N (mg./l.)	1·2	0·6
Total N (mg./l.)	1·2	0·6
NaCl (%)	0·01	0·49
pH	7·9	7·8
Free Cl_2 (mg./l.)	0·05	0·3
Combined Cl_2 mg./l.	0·2	0·5

ties of the water disappeared almost completely even with 1 mg. Cl_2/l. added; 2 mg. Cl_2/l. as NH_2Cl produced irritation, whereas 2 mg. Cl_2/l. as HOCl had no significant irritant effect.

Variation of pH between 7 and 9 showed no significant influence.

Different individuals showed, however, different sensitivities to irritation of the eye. One person, for example, had to be excluded from the experiments because of extreme sensitivity.

QUESTIONNAIRE STUDY

Material and methods

In order to test the hypothesis developed in the laboratory experiments that the saline concentration of swimming-pool water was of importance in the prevention of eye irritation, a limited questionnaire survey was performed in two indoor public baths, one with and the other without saline in the water. The chemical characteristics of the water at the two baths is given in Table 3. The pools are both 25 × 14 m. and have about 400–500 visitors every day. The water treatment process is $AlSO_4$-coagulation 2–3 times a week and filtration through sand. The saline water comes directly from the Baltic.

During one afternoon all persons above the age of 12 who used the pool were asked to complete a questionnaire concerning eye irritation, swimming habits and duration of swim. A total of 207 interviews were performed in the bath with tap-water and 161 in the bath with saline water. Very few respondents refused to take part in the investigation.

Results

The swimming habits for different age groups in the two baths are given in Table 4. It is seen that the proportion of younger swimmers was larger at the saline bath. In both baths the younger age groups were found to swim more frequently under water, dive, have the eyes open under water and swim for longer periods. In the saline bath more than 30 min. was spent in the pool by a higher proportion of both age groups than in the bath without saline. These results show that age and length of time in pool are two main determinants for the exposure of the eye to water.

The proportion of respondents in various age groups reporting eye irritation for

Table 4. *Swimming habits of two age groups at pools with and without saline*

	Tap-water		Saline water	
	Age 12–15	Age 16–72	Age 12–15	Age 16–72
Number of respondents ...	54	153	70	91
Percentage who:				
Swim under water	87	41	90	36
Dive	85	43	87	31
Open eyes under water	72	41	89	45
Stay in pool for				
Less than 30 min.	15	72	4	58
More than 30 min.	85	28	96	42

Table 5. *Number of respondents in different age groups, and the proportion reporting eye irritation in baths with and without saline*

	Tap water		Saline water	
Age	Number	Irritation (%)	Number	Irritation (%)
12–15	54	63	70	76
16–72	152	64	88	29

Table 6. *Relation between proportion of swimmers reporting eye irritation and time spent in pool*

	Tap water		Saline water	
Time in pool	Number	Irritation (%)	Number	Irritation (%)
< 30 min.	119	61	55	24
> 30 min.	87	66	106	62

the two baths is shown in Table 5. It is seen in the table that a higher percentage of the respondents recorded irritation in the bath without saline, except for the youngest age group where the extent of irritation was slightly higher in the saline bath. The proportion of respondents recording irritation of long duration was 41 % for the non-saline bath as compared to 31 % for the saline bath.

Table 6 shows the relation between length of time spent in the pool and irritation. It is seen in the table that the difference in extent of irritation between the two baths was present only among respondents spending less than 30 min. in the pool. For those spending a longer time in the pool, no difference in the extent of eye irritation was shown.

DISCUSSION

The method used to evaluate eye irritation in the laboratory experiments involved a relatively short-term exposure. The irritation was evaluated by means of the subjective impression of the person tested. With the pair comparison technique, however, the probability that methodological factors have influenced the results

are diminished. The present experiments comprised relatively few persons in the test groups, but as the results are consistent certain conclusions can be drawn.

The results from the laboratory study demonstrated that the addition of 0·7% NaCl totally decreases the irritating effect of water or moderately chlorinated water on the eyes during a 1·5 min. exposure. For higher chlorine concentrations, however, a certain degree of irritation remains. The results indicate that NH_2Cl is more irritating than HOCl and Chloramine B.

The results also support the general observations by Mood *et al.* (1951) that an increase in chlorine concentration will cause an increase in irritation. The concentration at which the irritation increases was not, however, the same. The finding that pH is a determinant for eye irritation is not supported by the present results.

The questionnaire survey performed to study the extent of irritation in saline and non-saline baths should only be regarded as preliminary and must be followed by larger-scale investigations with an experimental design before any final conclusions can be drawn. Certain data from the study are, however, of interest in evaluating the importance of the saline content of swimming-pool water.

Concerning swimming habits, a clear difference was found between young and old age groups. As might be expected, younger groups spent more time in the water and kept their eyes open under water to a larger extent. The results show that the eye irritation in the older group was considerably less in the saline water.

The decrease of eye irritation due to salinity was found to be closely correlated with the time spent in the pool. When the time was less than 30 min. a difference was found between the saline water and the non-saline. When the time spent in the pool exceeded 30 min. the saline provided no protective effect. This explains why no protective effect against eye irritation was found in the young age group. The saline content was, however, only 0·5%. A higher degree of protection might be expected with 0·7–0·8% NaCl.

The results from the present laboratory and field studies indicate a possibility of reducing eye irritation due to swimming-pool water exposure. If 0·7% NaCl is added to the water and the exposure time is limited to less than 30 min. at a time, there is reason to believe that less eye irritation will occur.

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Sonne dysentery in day schools and nurseries: an eighteen-year study in Edmonton

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SUMMARY

A study of Sonne dysentery infections in 19 primary and 11 secondary day-schools and 4 day-nurseries has been made over a period of 18 years in an urban area. Measures were taken throughout to try to prevent and control outbreaks. Sonne dysentery was not endemic in the school population and, even at times of high incidence, epidemics were localized within a few of the primary schools, usually in the spring or autumn terms. Incidence rates of Sonne dysentery were highest in primary schools with large infant departments and in nurseries. Secondary schools entirely escaped outbreaks.

The co-operation of head teachers was engaged for all precautionary measures in schools. A policy of immediate exclusion of suspected and infected children was useful in preventing and controlling school epidemics of dysentery. Toilet hygiene was often poor in schools with outbreaks, and this was found to be a profitable field for applying control measures. Infected kitchen workers were only occasionally involved.

Recommendations are given in the light of this study, and some reference is made to the more difficult problem posed by dysentery outbreaks in day-nurseries, where temporary closure may be the best policy. It is important that responsibility for infectious disease control in schools be clearly delineated in the reorganized health services of 1974. Teachers can play an important part in limiting infection.

INTRODUCTION

During the period 1951–68 general practitioners in the Edmonton district of Enfield in London were encouraged by the medical officer of health to seek a laboratory diagnosis for cases of gastroenteritis. Contacts were followed up by the public health department and school teachers co-operated in case-finding. The bacteriological investigations were carried out at Edmonton Public Health Laboratory. Intestinal infections discovered in school-children during this period were recorded in a special file as part of the record-keeping routine of the laboratory.

The study reported here is concerned with Sonne dysentery infections in the school-children who constituted the index (i.e. first) cases in their household and who are assumed to have acquired their infection outside the home. These children fall into two groups. Those who were first seen by their family doctor as an acute

case of diarrhoeal illness were grouped as *GP index cases*. The remainder, described as *MOH index infections*, were children diagnosed by the medical officer of health either after they had been sent home from school suffering from diarrhoea or vomiting or after the investigation of contacts of known cases of dysentery. Infected school-children who were home contacts of known index cases are not included since they may well have acquired infection in the home.

From 1953 onwards this study formed part of a larger survey of all dysentery in the borough of Enfield, which has already been reported (Thomas & Tillett, 1973). That survey showed that primary schools and nurseries were foci for the spread of dysentery in the community. Some of the survey results will be referred to in this report.

INCIDENCE OF SONNE DYSENTERY

In the general Enfield survey, where the mid-period population was 273,857, the average annual incidence rates per 10,000 population of Sonne dysentery index infections were 21·2 for pre-school children, 44·8 for primary-school-children aged 5–10 years, 3·8 for secondary-school-children aged 11–14 years and 1·1 for adults. It appears from these rates that dysentery is a disease of primary schools and is only a minor problem in secondary schools. Only a small proportion of pre-school children in this population attended nurseries, but within this small number many cases of dysentery were observed.

In the local study of Edmonton schools covering the years 1951–68 a total of 1088 Sonne dysentery index infections were found in children attending 19 primary schools. The district had just under 100,000 inhabitants and these schools had a mid-period population of 7667 children. There were 605 (56 %) GP index cases and 483 (44 %) MOH index infections. Of the latter group three-fifths of the children admitted to symptoms. Thus, there was an average annual incidence rate of primary schoolchild index infections of 78·8 per 10,000, which was even higher than that of 44·8 found in the same age group in the Enfield survey. Edmonton is more densely populated than the other areas of Enfield.

Distribution of cases in time

Two hundred and sixty-nine (25 %) of the index infections were diagnosed in the months September–December during the autumn term or Christmas holiday; 642 (59 %) were diagnosed in January–April during the spring term and Easter holiday; 177 (16 %) were diagnosed in the summer months May–August, more than nine-tenths of these before the summer holiday.

Incidence varied greatly from year to year and followed no apparent pattern. The school year was measured from September to August and the annual number of infections found in any of the 19 primary schools ranged between 1 and 135. In 4 school years fewer than ten infections were found and in 5 school years more than 100. Sonne dysentery did not therefore appear to be endemic in the schools.

This was further substantiated when outbreaks were considered. A large outbreak was defined as 10 or more index infections in any one term at any one school and a small outbreak as 5–9 infections. No outbreaks were observed in the

Table 1. *Numbers of outbreaks and sporadic incidents observed in* 19 *primary schools during* 18 *years and the numbers of children affected*

	Large outbreaks	Small outbreaks	Sporadic incidents	
Number observed in the 19 schools	29	14	148	
Total number of infections involved	763 (70 %)	95 (9 %)	230 (21 %)	1088

Table 2. *School terms during which Sonne dysentery was diagnosed in any of the* 19 *primary schools*

	Number of school terms			
	Autumn	Spring	Summer	Total terms
Large outbreaks in one or more school	7	10	3	20
Small, but no large outbreaks in progress	—	1	3	4
Only sporadic infections	7	5	9	21
No infections found in any schools	4	2	3	9
	18	18	18	54

11 secondary schools in the area, but 29 large outbreaks were observed in 13 of the 19 primary schools and accounted for 70 % of the 1088 total infections in that age group (Table 1). Fourteen small outbreaks in ten primary schools accounted for a further 9 % of total infections. No school was entirely free from sporadic cases throughout the 18-year period, although four schools escaped outbreaks.

Table 2 shows the distribution of outbreaks and sporadic cases over the school years. Outbreaks were in progress during 24 of the 54 school terms studied and sporadic infections were found during a further 21; nine terms were free of infection. Outbreaks were unusual in summer terms and the sporadic cases were often the tail end of outbreaks in the previous term. During the greater part of the study (i.e. 30 terms) there were no outbreaks in any of the primary schools.

Epidemics were localized, and even when large outbreaks were in progress by no means all the schools in the area were infected. Table 3 shows the average number of schools with small outbreaks, with sporadic or with no infections, during terms when large outbreaks were in progress. In one term (Spring 1962) there were as many as three large outbreaks; nevertheless, 9 of the 19 schools had no index infections that term. Whatever the number of large outbreaks in a term a large proportion of the schools remained wholly unaffected. In only two terms were less than half the schools free from known infection and these were the first term of the study when there were only four schools and the spring term of 1967 when there were eight schools free from infection.

The largest number of index infections found in one school in one term was 63, of which 55 were MOH index infections. The largest proportions of school populations found as index infections in outbreaks were 15 % in one outbreak in the

Table 3. *Primary schools affected by Sonne dysentery: distribution of cases in 19 schools during 54 terms*

Number of schools affected by large outbreaks	Total terms in which this number of large outbreaks was observed	Average number of schools during these terms with:		
		Small outbreaks	Sporadic cases	No infections
0	34	0·1	2·1	16·7
1	12	0·3	2·4	15·3
2	7	0·9	5·6	10·6
3	1	0	7	9

autumn of 1962 and 16% in an outbreak which lasted through the autumn and spring terms of 1967/8 in a school at the border of the area. Including sibs, 18% of the latter school population was found infected. This school was the subject of a special report (Thomas, Haider & Datta, 1972). Fifty-three per cent of cases found during outbreaks were MOH index infections, whereas 87% of sporadic cases were GP index cases detected by their family doctors. The remaining 13% of sporadic cases were discovered at home after they had been excluded from school because of diarrhoea or vomiting.

Distribution of cases between schools

The size of the schools ranged from 87 to 932 places with an average of 404. Three schools took only juniors (8- to 10-year-olds) and in the remainder just under half of the places were filled by infants (5- to 7-year-olds). The number of infections in a large school would be expected to be higher than those in a small school with fewer children at risk. This was observed. However, the infection rate per 100 pupils would be expected to be the same in all schools, all things being equal. The correlation between average annual infection rate and the number of infant places in each school was looked at and then the correlation between rate and the number of junior places. As the numbers involved were not normally distributed random variables, the correlations were evaluated by the ranking method of Kendall (1962).

Size of infant department

The correlation between the size of *infant* department and the infection rate was 0·35 and was statistically significant ($P < 0{\cdot}02$), whereas that between infection rate in a school and the size of the *junior* school department was 0·15 and was not significant. Fig. 1 shows the mean and range of incidence rates at groups of schools according to the size of their infant departments.

There were three schools in the study which took junior children only. Although one experienced an outbreak (Thomas *et al.* 1972) all three of these schools were free from known infection during all but two of the school years studied. This was a record achieved by only two other primary schools and these had small infant departments of 120 and 157 places.

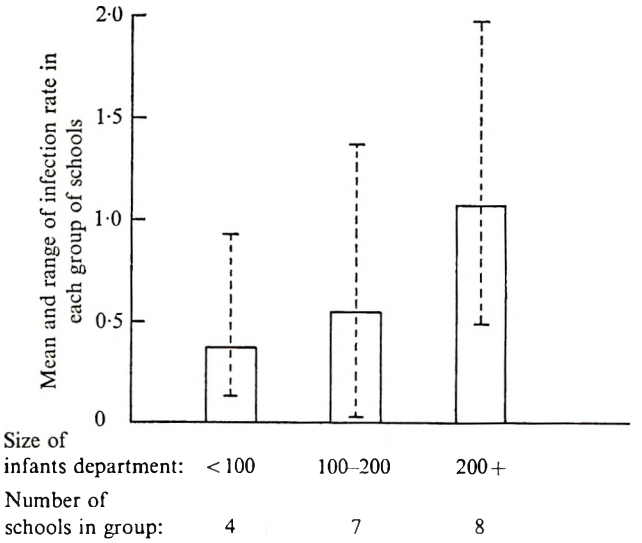

Fig. 1. Annual Sonne dysentery infection rate per 100 pupils in schools according to the size of their infant departments.

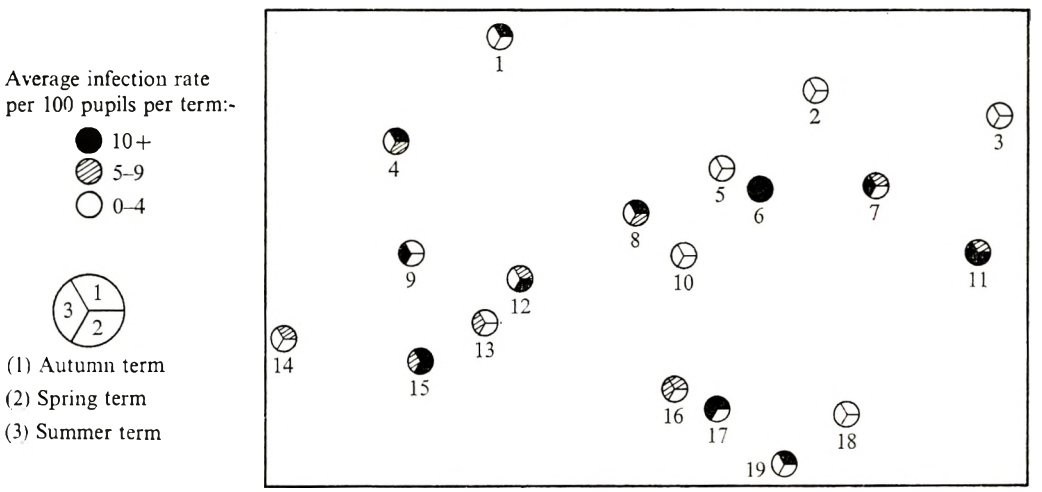

Fig. 2. Average Sonne dysentery incidence rates according to term and relative location of primary schools in Edmonton.

New buildings

The age of the buildings varied, nine schools being built before 1914 and five after 1945. No association was found between the age of the premises and the incidence rate of Sonne dysentery, even when allowance was made for the relative sizes of the infant departments. Four schools were rebuilt between 1950 and 1966 and it was disappointing to note no improvement in attack rates of dysentery – three of the four schools had slightly higher rates during the 7 years after rebuilding compared with the 5 years before rebuilding.

The 19 schools were spread over an area of about $3\frac{1}{2}$ square miles. In Fig. 2 they are shown in their relative positions but the compass reference has been

omitted. This figure shows the average incidence in each school during autumn, spring and summer terms. Schools tending to have high incidence at certain times of the year are not obviously clustered. Neither was a consistent pattern of spread of dysentery through the area seen when maps for individual years were studied.

The number of infants under 8 years old in a school was the *only* factor found which appeared to distinguish between schools with high or low dysentery infection rates. It was noted that the average number of outbreaks at 11 schools with under 200 infants was 1·1 and at the eight schools with over 200 infants was 3·9. This difference was highly significant ($P < 0{\cdot}001$).

OBSERVATIONS IN SCHOOLS WITH SONNE DYSENTERY OUTBREAKS

Visits to schools usually revealed bright and cheerful classrooms even in old buildings, but the maintenance of the toilets was never quite satisfactory in schools experiencing outbreaks of dysentery. The toilet equipment varied from modern to appalling. Frequently there was visible faecal soiling, including soiled toilet rolls. Also observed were situations where there were no accessible basins, blocked basin outlets, shared towels and no supervision.

School meals services were centrally organized and were not the source of any trouble. Some school servery kitchens, however, were poorly equipped. Occasionally servery staff, probably victims of an outbreak, were found to be infected. Children in some schools helped with the food serving and were seen to handle dishes and cutlery. Kitchen staff were also found to be helping in the cloakrooms.

Children were seen sucking their fingers and thumbs, sharing sweets and passing round chewed pencils and rubbers.

MEASURES TAKEN TO CONTROL AND PREVENT OUTBREAKS

Control

At the first signs of plural cases of dysentery a school was visited by a member of the local health department and a representative from the laboratory. This meant an immediate visit if two or more cases were diagnosed from the same school within 10 days. With the co-operation of the head teacher an inspection of the premises was carried out, together with appropriate bacteriological sampling. Inspection started in a classroom in order to praise and proceeded to the inevitably unsatisfactory lavatories. Arrangements were made for supervision of toilet hygiene, issue of individual paper towels, and a supervised disinfectant hand-dip after toilet, before meals and on re-entry to the classroom. Hand-dipping was found valuable because it focused attention on the state of the hands, giving the supervisor an opportunity to check that hands had been well washed beforehand. Even before fairly effective disinfectant solutions had been introduced this measure proved useful in checking the spread of infection. Frequent cleaning of toilet premises by attendants wearing rubber gloves was also advised.

Standing arrangements to exclude from schools any pupils with gastroenteritis symptoms, together with their siblings, pending negative bacteriological reports

on their faeces specimens were enforced. These children were re-admitted only with the written permission of the M.O.H.

If new cases continued to arise for more than 1 week after the first visit to the school, screening of the kitchen staff was carried out and any infected persons were excluded until three consecutive faeces specimens, collected on different days and at least 3 days after completing any antibacterial treatment, had been reported negative by the laboratory. If an outbreak reached large proportions, closure of severely affected classes was sometimes necessary. Sometimes two infected classes were found to be sharing the same toilet facilities – for example a boys' lavatory. No child would be re-admitted to these classes until they had negative bacteriological reports. In the meantime there was thorough cleaning of the premises.

Antibacterial drugs were not useful in the control of infection. Indeed, in the majority of cases studied *Sh. sonnei* persisted in the stools after appropriate drug treatment (Thomas & Tillett, 1973; Thomas *et al.* 1972).

Prevention

Preventive exclusion of gastroenteritis suspects was policy in the schools regardless of whether dysentery was currently prevalent. Good communication with the head teacher was important. Schools kept a stock of outfits for collecting specimens from children with symptoms of gastroenteritis. Such children were excluded from school by their teacher and given a faeces container and a printed envelope form for supplying name, age, address and school to the M.O.H. They were not re-admitted until a negative report had been sent from the laboratory to the M.O.H. and public health department.

It was considered that these measures for the prevention and control of outbreaks had a good effect. Despite intensive efforts to examine specimens from all suspects, index infection was never confirmed in more than 16% of the school population during any outbreak. These proportions do not include infected sibs. Nevertheless they are considerably lower than the two-thirds usually quoted for an uncontrolled outbreak (Annotation, 1966). A large part was played by the policies of school exclusion and screening of suspect contacts since over half the infections in school outbreaks were *MOH index infections.*

DAY-NURSERIES

Only one of a number of day-nurseries in the area continued throughout the period of study. This unit, of about 55 places, suffered four dysentery outbreaks, the largest of which affected 28 (51%) of infants. Every nursery was affected at some time.

Prevention of outbreaks of dysentery in day-nurseries was attempted by screening new entrants and children returning from residential institutions or hospitals, as these were the children who often introduced infection. It was usually practicable to screen these children before arrival, but occasionally specimens were taken on the first day and the children segregated until cleared. Any child attending a nursery who developed diarrhoea or vomiting was isolated and then excluded until bacteriologically cleared.

The procedure described for controlling outbreaks in primary schools was not found adequate at day-nurseries or nursery classes. Here prompt closure was more effective. The nursing hygiene was good in these institutions, but could not prevent case-to-case infection between incontinent toddlers. If two or more cases of dysentery were confirmed it became the practice to close the nursery for 3 days and wash it down with phenolic disinfectant. Thereafter staff and children could be re-admitted, provided that they had a minimum of two negative reports on faeces specimens. Observations were made in Edmonton between 1950 and 1954 of five nursery outbreaks where this closure procedure was followed, and four outbreaks where there was no closure but merely exclusion of infected persons. In the former a total of 168 children lost an average of 15·7 days attendance and 46 % became infected, and in the latter 154 children lost 18·8 days each and 63 % became infected. In the nurseries practising closure the premises were 'infected' on average for $8\frac{1}{2}$ days and a mean of 5·0 specimens were examined per head compared with 30 'infected' days and 7·5 specimens tested per head at the nurseries practising exclusion. Although it cannot strictly be concluded that these differences resulted from the differences between procedures, a policy of closing infected nurseries for a period of 3 days was continued because of the apparent benefit in cost of time and effort. The nursery supervisors were given direct access to the laboratory service for testing faeces specimens and they were responsible for isolation and exclusion.

SEASONAL PATTERN OF INFECTION

To the pertinent question 'Where does dysentery go in summer' we would answer that we have observed a link between dysentery outbreaks and infant-school toilet facilities. Dysentery bacilli deposited on surfaces survive longer in cold dark damp conditions than in warm light dry surroundings. The degree of contamination of lavatory accommodation by any one infected person is therefore likely to be greater in winter. The long summer vacation eliminates opportunity for cross-infection by congregation in communal toilets and interrupts the chain of transmission. In the autumn term a large number of 'virgin' infants, susceptible to dysentery, arrives at school for the first time and, if infection is offered by a convalescent excreter or carrier, a build-up of infected children can develop, sometimes quite rapidly. The Christmas holiday is not long enough to interrupt this significantly. By Easter, outbreaks tend to dwindle.

RECOMMENDATIONS AND DISCUSSION

Several recommendations arise out of this study of Sonne dysentery. Primary-school toilets should be equipped with foot-flushes, warm running water in unplugged basins, drip or powder soap and paper towels or blowers. Toilet use and hand washing should be supervised by special staff *not* involved in meals service. The teachers should be encouraged to exclude suspects and to re-admit them only after bacteriological clearance. During this study outbreaks were observed to follow the failure to exclude children who had recovered from an undiagnosed

gastroenteritis but who were subsequently found to be excreting *Sh. sonnei*. Also some school class outbreaks appeared to be uncontrolled by the methods described, until symptomless excreters were detected and excluded.

Primary schools should be kept to a manageable size, especially with regard to the size of their infant department (perhaps less than 200 children under 8 years). Protection of older children could be achieved by keeping them separate from the infants, who are usually responsible for the spread of the disease. Supervision could then be concentrated on the susceptible infants.

The advice given in the 1971 Memorandum on Infectious Diseases in Schools (D.E.S., 1971) and in the guide issued by the Society of Medical Officers of Health and the Public Health Laboratory Service (1970) is not strict enough. In the former memorandum we would think it safer if bacteriological clearance were to be required for children under 11 years old (p. 14), instead of leaving this to the discretion of the M.O.H. or the principal school medical officer. In the latter guide we would like to see children in primary schools included with the younger children in the special category of 'Those most likely to transmit infection' (p. 197), as this would exclude such children from returning to school until free from infection.

Children of pre-school age in institutions present even greater problems in dysentery control. If closure is possible, this appears to be the quickest method of ending an outbreak. The development of more day-nurseries demands careful planning with this problem in mind.

Since this study the numbers of notifications of dysentery reported by the Registrar General fell sharply in 1969 and have remained at a figure which is about half that which was being reported previously. However, the amount of dysentery and gastroenteritis reported by the Royal College of General Practitioners Research Committee has increased during these years. Therefore a fall-off of interest in notification may account for part of the drop in the Registrar General's Reports. It is interesting to note in this connexion that the notifications and isolations of bacillary dysentery fell between 1946 and 1950 only to rise subsequently to unprecedented heights. Outbreaks in institutions continue to come to the attention of the Public Health Laboratory Service and there is no firm evidence that the disease is on its way out. Although Sonne dysentery is usually a mild disease the Enfield survey showed that 4 % of GP index cases were admitted to hospital and nearly half the remainder had cellular stools. The mortality rate for notified cases is about 0·1 % in England and Wales.

It seems likely that Sonne dysentery will continue to cause primary-school and nursery outbreaks. Indeed other gastroenteritis infections, such as Flexner and Shiga dysentery and possibly cholera, may cause school outbreaks in the future and so call for a similar approach to control and prevention. Since these can be differentiated with certainty only by laboratory methods, it is important to maintain bacteriological surveillance of all infective gastroenteritis. This would seem possible given active co-operation between laboratory, medical officers of health and their successor departments, general practitioners, nursery supervisors and head teachers. It is to be hoped that the reorganized health services in 1974 will continue to give clear responsibility within the local health department for the

control of dysentery. The importance of a clear link between health department, school teachers and nursery supervisors must be realized.

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Nasal immunoglobulin responses in acute rubella determined by the immunofluorescent technique

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SUMMARY

The indirect immunofluorescent technique has been used to study the specific immunoglobulin responses in nasal secretions from ten adults with acute rubella. Titres of IgA antibody in nasal washings usually exceeded those of IgG, but both types of antibody were detected in all patients. They appeared a few days after the rash, reached maximum titres during the second week and then declined. IgA antibody was no longer detectable after 47 days and was not detected at all in nasal washings from adults who had experienced rubella in the past. Low titres of IgG antibody persisted in some patients for longer than IgA and traces of IgG were found in nasal washings from a minority of adults with a past history of rubella. Nasal antibodies in acute rubella are therefore transient and unlikely to take part in resistance to reinfection.

In sucrose-density gradients nasal IgA antibody sedimented more rapidly than IgG and there was little overlap between these two types of antibody. IgA antibody in serum was more heterogeneous; it was found in nearly all the fractions which contained IgG antibody and in many of those which contained IgM.

INTRODUCTION

In many viral infections antibody appears at mucous surfaces and may be found in nasal and other external secretions (Artenstein, Bellanti & Buescher, 1964). Studies in which nasal washings have been fractionated by gel filtration and by centrifugation on density gradients have shown that secretory antibodies consist principally of IgA, often with lesser amounts of IgG, and that both components may have antiviral activity (Bellanti, Artenstein & Buescher, 1965; Rossen *et al.* 1966; Alford, Rossen, Butler & Kasel, 1967). Secretory IgA is predominantly in the 11 S dimeric form and is produced locally by plasma cells in the lamina propria underlying the mucosa of the respiratory tract and intestine (Butler, Rossen & Waldmann, 1967; Rossen *et al.* 1967; Tourville, Adler, Bienenstock & Tomasi, 1969).

In infections with rhinoviruses and parainfluenza viruses, in which only superficial replication occurs, resistance to reinfection is related more closely to the presence of secretory antibody than to the titre of antibody in the serum (Cate *et al.* 1966; Smith, Purcell, Bellanti & Chanock, 1966; Perkins *et al.* 1969).

In infections with viruses such as measles and poliovirus, in which viraemia occurs, circulating antibody plays a part in protection from clinical disease, but in the latter infection secretory antibody produced in the intestine and nasopharynx may also help by preventing the virus from establishing itself at the primary site of infection (Ogra & Karzon, 1971).

In rubella the mechanism of resistance is not clear. The acute attack is normally followed by the appearance of circulating antibody and by lifelong immunity, but the latter is not related solely to serum antibody titres which in immune persons cover a very wide range. Secretory antibody in rubella has received little study except by Ogra *et al.* (1971), who demonstrated specific IgA and IgG antibodies in nasal washings from children with acute rubella by means of the radio-immunodiffusion technique. We have used the indirect immunofluorescent technique to study specific immunoglobulin antibodies in nasal washings from adults with acute rubella, patients with maculo-papular rashes not caused by rubella, and healthy volunteers who had experienced rubella in the past.

MATERIALS AND METHODS

Patients with acute rubella

Specimens were obtained from one male and nine female patients, aged 21–33 years, who were suffering from acute rubella. Six of the nine females were pregnant. Altogether 49 specimens of nasal washings and 35 specimens of serum were obtained from patients in this group at various times after the onset of the rash. The diagnosis was confirmed by a rise of at least fourfold in the haemagglutination-inhibition (HAI) titre in all cases and by isolation of rubella virus from the nasal washings in nine cases.

Adults with serum antibodies due to past infection with rubella

Single specimens of nasal washings were taken from 11 male and 11 female healthy adult volunteers aged 18–45 years, all of whom possessed rubella HAI antibody. Four of the males had suffered from serologically confirmed rubella about 1 year previously. The other volunteers gave no recent history of the disease and were presumed to have experienced it in the more distant past.

Patients with rashes not due to rubella

Eleven patients, aged 13–27 years, were studied because they had clinical syndromes consisting of a rubelliform rash, accompanied in some cases by enlargement of the cervical or occipital lymph nodes. Three patients were male. All the eight females were pregnant. Paired sera were obtained from all patients in this group. Paired specimens of nasal washings were obtained from eight patients, but

only a single washing was available from each of the other three (at 6, 8 and 12 days after the onset of the rash).

Collection and treatment of nasal washings

While the patient sat upright with the head extended, 5 ml. of phosphate-buffered saline (PBS) was instilled into one side of the nasal cavity through an infant feeding tube which was passed about 2 cm. into the nose. The patient then leaned forward and forcibly expelled the washings into a glass jar. This procedure was repeated on the opposite side and the washings from both sides were pooled. The sample was emulsified in a blender and volumes of 0·2 ml. were inoculated into cultures of RK13, secondary rhesus monkey kidney, HEp2 and WI38 cells for virus isolation. The remainder was left overnight at 4° C. to allow all globulin to be fully eluted from any residual shreds of mucus. The specimen was then centrifuged at 2000 rev./min. for 20 min. and the supernatant inactivated at 56° C. for ½ hr. Gentamicin (100 μg./ml.) was added to the final material, which was then stored at −20° C. until required.

Nasal washings were tested for rubella HAI antibody and were examined by the indirect immunofluorescent technique for the presence of specific IgG, IgA and IgM antibodies. In the detection of antibodies to rubella the washings were used to stain cover-slip preparations of BHK21 cells infected with rubella virus. Specimens from five patients with acute rubella were also tested for antibodies to respiratory syncytial (RS) virus, for which purpose cover-slip preparations of the same cells infected with RS virus were used (see below).

The total IgA concentration was measured by single radial diffusion in commercial immuno-plates designed for the measurement of low concentrations of serum IgA,* using solutions of 7 S IgA as standards. Assuming that the IgA in nasal washings has a sedimentation constant of 11 S, this method could be expected to give results which would be about 70% of the true values (Hobbs, 1970). In fact many of the precipitation rings were too narrow for accurate measurement and have been recorded as '< 4 mg./100 ml.'. For this reason we have not tried to adjust the readings to give a truer index of 11 S IgA concentration, nor have we altered the specific IgA titres to allow for differences in the total amount of IgA. The results are nevertheless of some value as a guide to the degree of variation in the concentration of IgA. The albumen content was also measured in Hyland immuno-plates.

Concentration of the globulins in selected nasal washings was carried out before centrifugation on sucrose-density gradients. We initially attempted to concentrate individual specimens by dialysis against 'Carbowax' and by treatment with 'Lyphogel', but the results were inconstant and the increase in total IgA concentration was usually less than proportional to the reduction in volume. Better results were obtained by precipitating the globulins with ammonium sulphate and then redissolving in a smaller volume of PBS. Before precipitation the washings were absorbed with chick red cells for 1 hr. at 4° C. in order to remove non-specific agglutinins. One volume (7 ml.) of saturated ammonium sulphate at pH 7·0 was

* Obtained from Hyland Division of Travenol Laboratories Limited, Thetford, Norfolk.

then added slowly to an equal volume of washings while the latter was agitated continuously by a magnetic stirrer. The mixture was allowed to stand overnight at 4° C. and the resulting fine precipitate was deposited in an ultracentrifuge at about 25,000*g*. The supernatant was removed and the deposit was resuspended in PBS to one eighth of the original volume. No reprecipitation or dialysis was carried out. The resulting solution was clarified by light centrifugation and 0·5 ml. of the supernatant was layered on top of a sucrose gradient. Comparisons of specific IgA titres showed that an increase in concentration of about fourfold was produced by this means.

Cover-slip preparations for fluorescent staining

Cover-slip cultures of BHK21 (clone 13) cells infected with the Judith strain of rubella virus were prepared by the method described in previous work (Cradock-Watson, Bourne & Vandervelde, 1972). For the preparation of cultures of the same cells infected with RS virus the method was modified in the following manner. Pieces of cover-slip 5 mm. in width were placed in $4 \times \frac{1}{2}$ in. tissue culture tubes to which 1 ml. volumes of growth medium containing between 200,000 and 250,000 cells were then added. The tubes were incubated at 37° C. in a sloped position for 24 hr. when they were infected by replacing the growth medium with a suspension of the Long strain of RS virus in maintenance medium. The virus was stored in liquid nitrogen as a stock of known titre and was diluted for use so that it had a final titre of about $10^{3 \cdot 5}$ TDC50 per ml. when titrated in HEp2 cells. The infected tubes were reincubated at 35° C. for 48 hr., after which the cover-slips were removed, rinsed in PBS, fixed in acetone and finally allowed to dry in air. Uninfected cultures were prepared for use as controls.

Fluorescent staining

Cover-slip preparations were stained by the indirect immunofluorescent technique as previously described (Cradock-Watson *et al.* 1972). Each serum received two preliminary absorptions at a dilution of 1/8 with washed BHK21 cells in order to reduce non-specific fluorescence. Nasal washings were not absorbed with these cells before staining. After treatment with serum or with nasal washings the cover-slips were washed in PBS and then stained with fluorescein-conjugated globulins prepared against human IgG, IgA or IgM (Wellcome Reagents Limited). Anti-IgG conjugate was not absorbed and was used at a dilution of 1/120. Conjugates prepared against IgA and IgM were absorbed once at a dilution of 1/8 with BHK21 cells and were used at dilutions of 1/32 and 1/48 respectively. A single batch of each type of conjugate was used throughout. The stained cover-slips were washed in PBS, mounted in glycerol and examined by quartz-halogen illumination, using an interference filter of the type described by Rygaard & Olsen (1969).

Haemagglutination-inhibition titrations

Sera were inactivated at 56° C. for $\frac{1}{2}$ hr., absorbed with kaolin, and titrated in WHO plastic trays by the method in routine use in the Manchester Public Health Laboratory (Thompson & Tobin, 1970). Nasal washings were not treated with kaolin but were absorbed with chick red cells and were tested in a similar manner.

Sucrose-density gradient centrifugation

A volume of 0·5 ml. of nasal washings, or 0·5 ml. of a 1/2 dilution of serum, was layered on top of a sucrose gradient extending from 12·5 to 37·5 % (w/v) which was then centrifuged at 35,000 rev./min. for 17 hr. About 12 fractions were collected after piercing the bottom of the tube. The presence of IgG and IgM in the fractions was detected by double diffusion in agar, using antisera specific for human IgG and IgM (Wellcome Reagents Limited). The concentration of IgA in each fraction was measured in Hyland immuno-plates, using solutions of 7S IgA as standards. Rubella-specific immunoglobulins in the fractions were titrated by the indirect immunofluorescent technique, and HAI activity was titrated in microtitre trays.

RESULTS

Microscopic appearances of fluorescent staining

The appearance of rubella-infected cells stained with nasal washings was similar to that produced by staining with serum. Fine fluorescent granules were seen in the cytoplasm of a proportion of cells in the preparation. Fluorescence was often brightest near the nucleus, but no nuclear staining was seen. In titrations of nasal washings and sera the numbers and brightness of individual fluorescent cells progressively diminished, and the end-point was taken as the last dilution at which specific fluorescence could clearly be seen. Some nasal washings, when tested undiluted for IgA, showed non-specific staining which could not be removed by prior absorption with BHK21 cells. This disappeared with increasing dilution and seldom interfered with the measurement of specific IgA. Non-specific staining in specimens stained for IgG was negligible. Apart from this, no consistent difference was observed between IgG and IgA staining. An example of fluorescent staining for rubella IgA antibody in a nasal washing is shown in Plate 1, fig. 1.

In BKH21 cells infected with RS virus the fluorescent material was cytoplasmic and consisted of particles ranging from relatively large round or oval bodies down to small granules and fine threads. No nuclear fluorescence was seen. An example of fluorescent staining for IgG antibody to RS virus in human serum is shown in Plate 1, fig. 2.

HAI antibody in nasal washings from patients with acute rubella

HAI antibody appeared in nasal washings from all patients in titres ranging from 8 to 64 (Table 1). It was first detected (in titres > 1) 3 days after the onset of the rash and reached its highest titres in 7–15 days. It then rapidly declined and only low levels of HAI activity persisted in those patients (cases 1, 2, 3 and 10) in whom follow-up was possible.

Nasal immunoglobulin responses in patients with acute rubella

Immunofluorescent staining showed that IgA was the predominant type of rubella antibody in nasal secretions taken in early convalescence (Table 1). IgA antibody was found in all patients. It was first detected 4 days after the onset of

Table 1. *Haemagglutination-inhibition and immunoglobulin antibody titres in nasal washings from ten patients with acute rubella*

Case no.	Days after onset of rash	Albumen concentration (mg./100 ml.)	Total IgA concentration (mg./100 ml.)	HAI titre	Immunoglobulin titre obtained by immunofluorescence: Rubella IgG	Rubella IgA	Respiratory syncytial virus IgG
1	1*	< 10	4·6	< 1	< 1	< 1	< 1
	3*	13	4·6	1	< 1	< 1	1
	8	15	5·9	32	16	64	2
	10*	13	6·4	16	2	32	2
	15	15	5·9	8	2	8	2
	17	< 10	4·6	1	1	< 1	< 1
	24	< 10	< 4	1	< 1	NSF†	< 1
	42	< 10	< 4	1	< 1	< 1	< 1
2	8*	29	10	64	32	256	2
	10*	12	< 4	16	8	128	< 1
	12*	12·5	4·1	16	2	16	< 1
	15	10·5	< 4	4	1	16	< 1
	20	11	4·1	4	1	4	.
	47	12	5·6	4	4	2	< 1
	168	< 10	< 4	1	2	< 1	< 1
3	1*	12	3·7	< 1	< 1	< 1	< 1
	10*	18	4·6	8	8	16	2
	21	< 10	3·7	2	1	2	< 1
	28	< 10	3·7	1	1	2	1
	56	< 10	4·3	1	1	< 1	< 1
4	0*	< 10	7·6	< 1	< 1	< 1	.
	7	< 10	8·8	8	2	8	.
	10	< 10	7·2	4	2	2	.
	14	10·5	5·2	1	1	< 1	.
	21	10·5	5·2	2	2	NSF	.
5	4*	11	5·2	8	< 1	4	1
	8	11	5·2	64	8	128	1
	11*	15	17	64	16	128	2
	16	21	34	32	16	32	2
	22	21	6·2	16	32	2	4
	30	11	5·2	4	2	< 1	1
6	1*	10	4·6	< 1	< 1	< 1	.
	5	11	4·6	2	< 1	8	.
	8	< 10	4·6	8	< 1	8	.
	15	< 10	5·5	16	2	32	.
7	3*	50	17	8	< 1	NSF	.
	7	11	5·2	16	1	16	.
	11	< 10	4·6	2	< 1	2	.
	24	< 10	4·9	2	< 1	< 1	.
8	1*	17	10·5	1	< 1	<1	.
	8*	21	13	16	2	2	.
9	5*	11	6·2	4	< 1	4	.
	8	10·5	7·7	32	1	32	.
	12	13	7·2	8	2	8	.
10	10	52	34	64	8	32	< 1
	15	11	4·9	8	< 1	4	< 1
	28	10	4·9	1	1	< 1	< 1
	35	10	4·9	< 1	< 1	< 1	< 1
	83	< 10	10	2	2	< 1	< 1

* Rubella virus isolated. † NSF = non-specific fluorescence interfered with reading.

Table 2. *Haemagglutination-inhibition and immunoglobulin antibody titres in sera from ten patients with acute rubella*

Case no.	Days after onset of rash	HAI titre	Immunoglobulin titre obtained by immunofluorescence: Rubella IgG	Rubella IgA	Rubella IgM	Respiratory syncytial virus IgG
1	1	< 20	< 8	< 8	< 8	2048
	3	60	64	64	16	.
	8	1280	1024	1024	1024	1024
	10	960	1024	512	256	.
	17	960	4096	512	64	1024
	24	640	1024	16	< 8	.
	42	480	2048	16	< 8	.
	143	320	2048	16	< 8	2048
2	1	< 20	< 8	32	< 8	1024
	8	1280	1024	512	< 8	.
	12	2560	4096	2048	< 8	1024
	15	2560	8200	1024	< 8	.
	47	1280	4096	< 8	< 8	512
3	1	< 20	< 8	< 8	< 8	2048
	10	640	1024	256	64	2048
4	0	< 20	< 8	< 8	< 8	.
	7	1280	4096	256	< 8	.
5	4	240	64	512	128	1024
	8	2560	1024	512	128	512
6	1	< 20	16	< 8	< 8	.
	5	480	128	1024	128	.
7	3	160	32	128	256	.
	7	1280	2048	1024	1024	.
	11	1280	2048	512	512	.
	24	1280	4096	128	128	.
	108	640	4096	< 8	< 8	.
8	1	< 20	< 8	< 8	< 8	.
	8	160	128	128	32	.
9	5	160	256	128	64	.
	8	640	1024	512	128	.
	12	1280	2048	512	128	.
10	2	< 20	8	128	< 8	1024
	10	1280	1024	4096	64	1024
	28	640	1024	128	< 8	.
	35	480	2048	< 8	< 8	2048

the rash, reached titres ranging from 2 to 256 in 7–15 days, and then rapidly declined. It was not detected after 47 days, but in cases 6, 8 and 9 the duration of IgA antibody was uncertain because of insufficient follow-up. Attempts to detect very low levels of IgA antibody by staining concentrated specimens proved unsatisfactory because of non-specific staining. The total IgA concentration showed only slight variation, except in a few individual instances (Table 1). Attempts to

Table 3. *Rubella antibodies in fractions obtained by centrifuging neat nasal washings on a sucrose-density gradient*

(Case no. 2, pooled washings 8 and 10 days after rash.)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion*		Immunofluorescence titre of rubella-specific immunoglobulin	
		IgG	IgA mg./100 ml.	IgG	IgA
1	< 1	–	< 4	< 1	< 1
2	< 1	–	< 4	< 1	2
3	4	–	< 4	< 1	16
4	8	–	5·8	< 1	64
5	8	–	7·4	< 1	128
6	4	–	< 4	< 1	8
7	4	tr	< 4	32	2
8	4	tr	< 4	32	1
9	2	–	< 4	2	1
10	< 1	–	< 4	< 1	1
11	< 1	–	< 4	< 1	1
Titre before fractionation	32	.	.	32	256

tr = trace.

* IgG was detected by double diffusion in agar. IgA concentration was measured in Hyland immuno-plates.

adjust the specific IgA titres to allow for differences in the total amount of IgA would not have altered the general pattern of IgA response.

IgG antibody was found in nasal washings from all patients in titres which were usually lower than those of IgA. It was first detected 7 days after the onset of the rash, reached titres ranging from 1 to 32 within 7–22 days, and then declined. A little was still detectable at 30 days in case 5, at 56 days in case 3, at 83 days in case 10, and at 168 days in case 2. In case 1, the only other patient in whom follow-up was possible, IgG antibody was no longer detectable at 24 days. In five patients (cases 1, 2, 3, 5 and 10) evidence of an approximate relationship between the titre of IgG antibody and the concentration of albumen suggested the possibility that nasal IgG might be derived from the serum and that its concentration in nasal washings might reflect either the quality of the specimen or the presence of inflammatory exudation. We sought other evidence for the transfer of serum IgG to the nasal secretions by examining washings from these five cases for the presence of specific IgG antibody to RS virus. However, although the serum titres of this antibody were of the same order as those of IgG antibody to rubella (Table 2) the nasal titres were less than those of rubella IgG and showed much less variation.

No nasal IgM antibody to rubella virus was detected in any patient in this group.

Nasal antibody in adults with serum antibody due to past infection

When undiluted washings were examined for IgA antibody non-specific fluorescence interfered with detection in three cases. No IgA antibody was detected in the other 19.

IgG antibody was detected in undiluted washings from one male volunteer who had experienced an attack of confirmed rubella one year previously. When concentrated washings were examined traces of IgG antibody were detected in four more males and one female, none of whom gave any recent history of the disease.

No IgM antibody was detected in any volunteer in this group.

HAI activity was detected in undiluted washings from three male volunteers, and traces of activity were detected in seven others, but no activity was detected in dilutions of 1/2 or greater. Three of the washings with HAI activity also contained traces of IgG antibody when examined after concentration.

Nasal antibody in patients with rashes not due to rubella

Nine patients in this group had stationary serum HAI titres in acute and convalescent sera, indicating rubella infection in the past. Two patients had no serum antibody and did not develop any during their illnesses. In one patient with serum HAI antibody a trace of specific IgG was detected in the first nasal washing (day 1) which also had an HAI titre of 4, but not in the second washing (day 8) which showed no HAI activity. No rubella-specific immunoglobulin was detected in nasal washings from any other patient in this group. One other patient had an HAI titre of 4 in a single nasal washing taken 12 days after the rash, but in all other patients in this group HAI activity was either absent or detectable only in undiluted washings.

No virus was isolated in cell culture from any of the patients in this group. Specimens from four patients were inoculated intracerebrally into suckling mice with negative results. No serological evidence of infectious mononucleosis, cytomegalovirus infection or toxoplasmosis was obtained, and the causes of the rashes in these eleven patients remained undiscovered.

Centrifugation of nasal washings on sucrose-density gradients

In three patients (cases 1, 2 and 5) high titres of specific IgG and IgA antibodies were found in nasal washings and we attempted to characterize these immunoglobulins further by centrifuging the specimens on sucrose-density gradients. Pooled nasal washings from case 2 (days 8 and 10) were examined before and after concentration. The results are shown in Tables 3 and 4 respectively. IgA sedimented more rapidly than IgG, and in unconcentrated washings almost no overlap was detected between these globulins. The effect of concentrating the washings was to produce an increase of at least fourfold in the titres of antibody. There was consequently some broadening of the zones in which each class of antibody was detected and some increase in the area of overlap between IgG and IgA, but no evidence that precipitation with ammonium sulphate caused any qualitative change in the sedimentation pattern.

Table 4. *Rubella antibodies in fractions obtained by centrifuging concentrated nasal washings on a sucrose-density gradient*

(Case no. 2, pooled washings 8 and 10 days after rash.)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion* IgG	Immunoglobulin detected by gel diffusion* IgA mg./100 ml.	Immunofluorescent titre of rubella-specific immunoglobulin IgG	Immunofluorescent titre of rubella-specific immunoglobulin IgA
1	4	–	< 4	< 1	< 1
2	8	–	6·6	< 1	< 1
3	16	–	7·4	< 1	32
4	16	–	14	< 1	128
5	⩾ 64	–	35	< 1	1024
6	⩾ 64	–	29	< 1	512
7	16	+	8·4	16	32
8	16	+	7·4	128	2
9	8	+	6·6	64	2
10	4	–	< 4	2	< 1
11	2	–	< 4	< 1	< 1
Titre before fractionation	2048	.	.	128	1024

* IgG was detected by double diffusion in agar. IgA concentration was measured in Hyland immuno-plates.

Table 5. *Rubella antibodies in fractions obtained by centrifuging concentrated nasal washings on a sucrose-density gradient*

(Case no. 5, pooled washings 8 and 11 days after rash.)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion* IgG	Immunoglobulin detected by gel diffusion* IgA mg./100 ml.	Immunofluorescent titre of rubella-specific immunoglobulin IgG	Immunofluorescent titre of rubella-specific immunoglobulin IgA
1	< 1	–	< 4	< 1	< 1
2	< 1	–	< 4	< 1	< 1
3	< 1	–	< 4	< 1	< 1
4	2	–	4·1	< 1	< 1
5	2	–	7·4	< 1	16
6	4	–	16	< 1	64
7	8	–	29	< 1	128
8	4	–	14	< 1	64
9	2	tr	5·8	8	< 1
10	4	tr	9·2	64	1
11	2	tr	< 4	16	< 1
12	< 1	–	< 4	< 1	< 1
13	< 1	–	< 4	< 1	< 1
Titre before fractionation	16	.	.	64	256

tr = trace

* IgG was detected by double diffusion in agar. IgA concentration was measured in Hyland immuno-plates.

Table 6. *Rubella antibodies in serum fractions obtained by centrifugation on a sucrose-density gradient*

(Case no. 2, 12 days after rash.)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion*			Immunofluorescent titre of rubella-specific immunoglobulin		
		IgG	IgA mg./100 ml.	IgM	IgG	IgA	IgM
1	2	–	< 4	–	< 1	< 1	32
2	4	–	< 4	+	< 1	4	128
3	4	–	< 4	+	< 1	64	64
4	8	tr	8·4	–	16	512	< 1
5	16	+	22	–	1024	2048	< 1
6	⩾ 64	+	33	–	1024	512	< 1
7	⩾ 64	+	29	–	1024	32	< 1
8	8	+	7·4	–	512	16	< 1
9	4	tr	< 4	–	32	8	< 1
10	16	tr	< 4	–	4	< 1	< 1
Titre before fractionation	2048	.			4096	2048	< 8

tr = trace.

* IgG and IgM were detected by double diffusion in agar. IgA concentration was measured in Hyland immuno-plates.

Table 7. *Rubella antibodies in serum fractions obtained by centrifugation on a sucrose-density gradient*

(Case no. 5, 8 days after rash.)

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion*			Immunofluorescent titre of rubella-specific immunoglobulin		
		IgG	IgA mg./100 ml.	IgM	IgG	IgA	IgM
1	4	–	< 4	–	< 1	4	32
2	8	–	< 4	+	< 1	8	64
3	4	–	6·6	+	< 1	16	64
4	4	–	8·4	tr	< 1	64	16
5	8	tr	9·2	–	8	512	< 1
6	32	+	25	–	512	256	< 1
7	⩾ 64	+	43	–	4096	256	< 1
8	⩾ 64	+	47	–	4096	32	< 1
9	32	+	23	–	1024	32	< 1
10	4	+	5·8	–	32	2	< 1
11	4	–	< 4	–	8	2	< 1
12	16	–	< 4	–	4	1	< 1
Titre before fractionation	4096	.	.	.	1024	512	128

tr = trace.

* IgG and IgM were detected by double diffusion in agar. IgA concentration was measured in Hyland immuno-plates.

Satisfactory results with specimens from cases 1 and 5 were obtained only after previous concentration, which again raised the antibody titre fourfold. In both cases the IgA antibody sedimented more rapidly than the IgG, with only slight overlap. The results from case 5 are shown in Table 5. The distribution of specific antibody determined by fluorescence closely matched the concentration of IgA determined in immuno-plates. Although no markers were used the results suggest that nasal IgA antibody in these cases was predominantly in the 11S dimeric form. HAI activity was associated with both types of antibody.

Serum immunoglobulin responses in patients with acute rubella

The titres of rubella antibody in the IgG, IgA and IgM immunoglobulin classes are shown in Table 2. Antibodies in all three classes increased virtually simultaneously. IgG antibody followed the same course as HAI antibody and reached titres ranging from 128 to 8200. IgA antibody was detected in all patients and reached titres ranging from 128 to 4096. IgM antibody was detected by fluorescence in eight patients and reached titres ranging from 32 to 1024. It was not detected by fluorescence in cases 2 and 4 when whole serum was examined but was detected by fluorescence in the heavy fractions of serum taken from case 2 on the twelfth day after the rash (Table 6). It was impossible to obtain serial samples from all patients but the results agree with previous findings that IgA and IgM antibodies are transient in acute rubella and that the presence of either may be an indication of recent infection (Bürgin-Wolff, Hernandez & Just, 1971; Cradock-Watson *et al.* 1972).

Centrifugation of sera on sucrose density gradients

Sera from case 1 (day 8), case 2 (day 12) and case 5 (day 8) all contained high titres of IgA antibody. We therefore centrifuged these sera on density gradients in order to compare the sedimentation behaviour of the IgA in the serum with that of the IgA in the nasal washings taken at about the same time. The sedimentation patterns of the three sera were similar and resembled those obtained in our previous work. The results from cases 2 and 5 are shown in Tables 6 and 7 and correspond in time to the nasal washings shown in Tables 4 and 5 respectively.

A large proportion of serum antibody detectable by fluorescence consisted of IgG, the distribution of which corresponded well with the presence of IgG as detected by gel diffusion.

IgA antibody detected by fluorescence showed a wider distribution in the fractions from serum than in those from nasal washings. Nearly all the serum fractions containing IgG antibody also contained IgA. The distribution of IgA antibody detectable in serum by fluorescence did not exactly match the total IgA concentrations but showed a shift towards the heavier fractions and extended into the IgM zone. The results suggest that the freshly formed serum IgA antibody in these patients was heterogeneous in composition, and may have contained dimeric and polymeric forms in addition to monomers.

IgM antibody was detected by fluorescence in the heavy fractions from all three

patients, although in case 2 it had not been detected by fluorescence when whole serum was examined.

DISCUSSION

Our results show that antibodies to rubella virus appear in the nasal secretions in adults with acute rubella but not in patients with rubelliform rashes due to other causes. The appearance of nasal antibody was not associated with rhinorhoea, the symptoms of which had disappeared from all patients several days before the antibody responses reached their peaks.

Nasal antibody was predominantly IgA which appeared a few days after the rash, reached a peak during the second week and then rapidly declined. IgA antibody was no longer found in acute cases after 47 days and none was found in specimens from normal adults who had experienced rubella in the past. Our results differ from those obtained by Ogra *et al.* (1971), who found that nasal antibody developed between one and two months after the rash and persisted with very little fall for at least a year.

The observation that nasal and serum IgA antibodies follow similar courses is consistent with the possibility that nasal IgA in rubella is derived from the serum. However, the difference in the sedimentation behaviour of nasal and serum IgA is more suggestive of the generally accepted view that most of the secretory IgA is in the form of an 11S dimer produced locally.

The origin of IgA in the serum in man is uncertain, but consideration of the relative numbers of IgG- and IgA-producing cells in peripheral lymphoid tissue has led to the suggestion that a significant amount of IgA produced in the mucosa in the form of 7S monomers and 10S dimers may diffuse via the lymphatics into the blood (see Tomasi, 1972). The results described here are consistent with this view because comparison of the distribution of virus-specific IgA with total IgA in density gradients suggests that a disproportionate amount of freshly formed IgA antibody sediments relatively heavily and may therefore be in dimeric or polymeric form.

Titres of serum IgG antibody rose rapidly and remained high whereas nasal IgG antibody, which appeared at about the same time, tended to decline to low levels or disappear altogether. The observation that nasal and serum IgG antibodies followed different courses suggests that most of the nasal IgG in acute rubella is locally produced. This conclusion is supported by the observation that nasal titres of IgG antibody to RS virus were low and showed comparatively little variation although the titres of IgG antibody to this virus in the serum were comparable with the titres to rubella.

HAI activity to influenza A2 virus was demonstrated in nasal washings by Alford *et al.* (1967) following experimental infection in human volunteers. The activity varied according to the total amounts of IgA and protein in the specimens and was considered to be a non-specific effect due to the presence of mucoproteins. In our work slight rubella HAI activity, which may well have been non-specific, was detected in nasal washings from a few individuals in the control groups. However, in the cases of acute rubella HAI activity and fluorescent antibody levels in

nasal washings followed similar courses. Moreover in density gradient fractions the HAI activity varied with the titres of specific IgG and IgA and it therefore seems likely that most of the HAI activity in nasal washings from acute cases was specific and attributable to both these types of antibody.

By comparison with some other viral infections nasal antibody in rubella appears to be short-lived. Smith *et al.* (1966) found that titres of nasal antibody to parainfluenza virus type 1 remained high for 5 weeks and that antibody was still detectable in some cases after 8 months. Cate *et al.* (1966) found that rhinovirus antibodies were still present in nasal secretions after 56 days with little evidence of fall in titre. Although reinfection with both these agents can occur, resistance to reinfection is probably closely related to the nasal antibody titre. In the cases of rubella described here nasal IgA antibody rapidly disappeared and only traces of IgG remained in a few patients. It seems unlikely, therefore, that nasal antibody helps to prevent reinfection. It is possible that it could take part in recovery from the acute infection but its efficiency is uncertain because in four of the patients described here rubella virus was isolated from the washings after nasal antibody had reached, or even passed, its peak.

Immunofluorescence has certain advantages in the study of secretory antibodies. It is sensitive and specific and allows the detection of immunoglobulin antibodies in secretions from individual patients without the need for fractionation. The method is independent of phenomena such as complement fixation, neutralization and agglutination, and free from the limitations which tests for these functions impose. It seems a promising technique for the study of secretory antibodies from other sites and in other infections.

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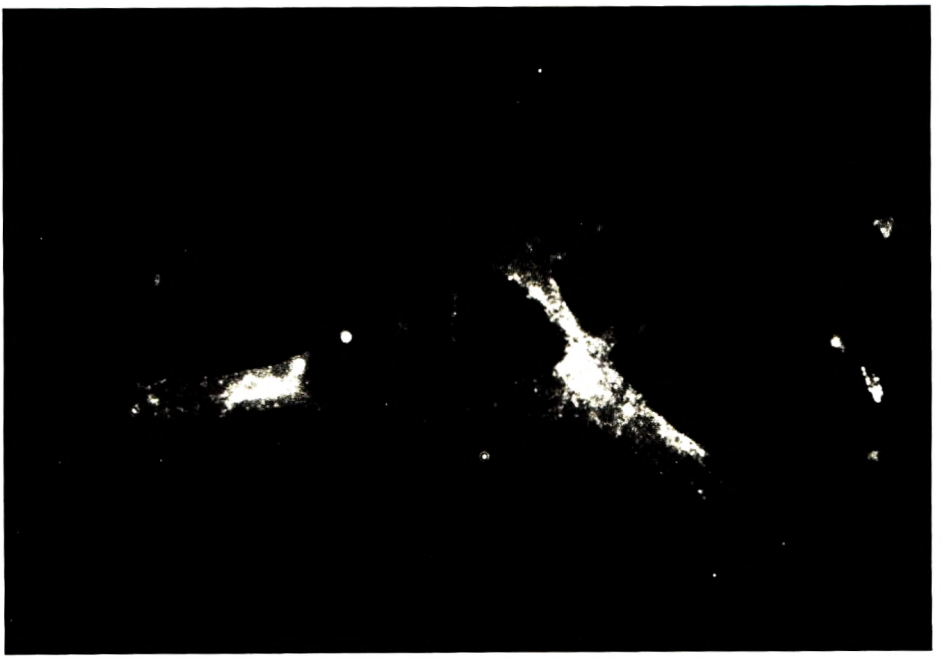

Fig. 1

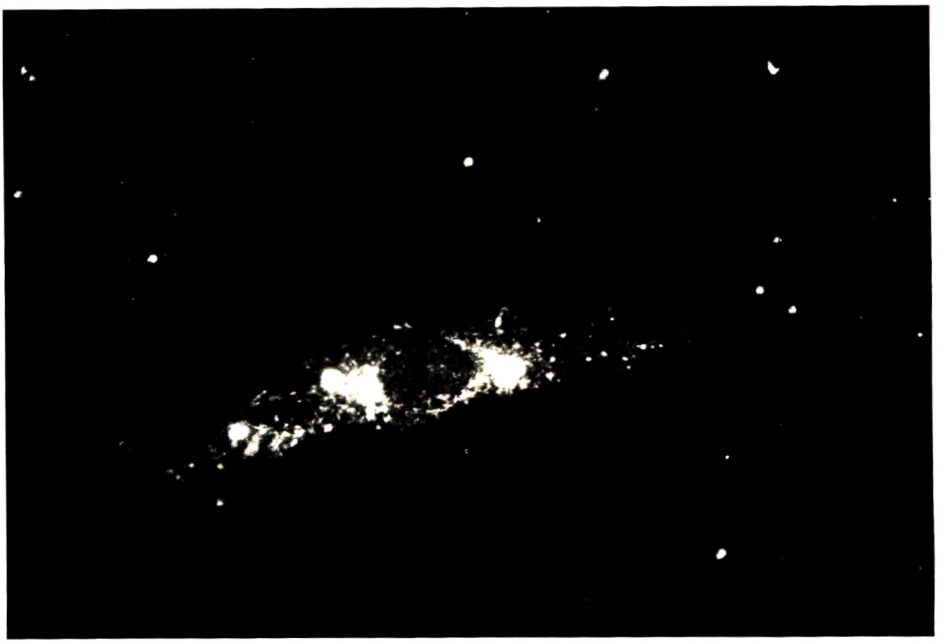

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Immunofluorescent staining of nasal IgA antibody to rubella. × 700. Nasal washings from case 2, 8 days after the rash, at a dilution of 1/16.

Fig. 2. Immunofluorescent staining of serum IgG antibody to respiratory syncytial virus. × 420. Serum dilution 1/40.

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Bacteriophage restriction in *Salmonella typhimurium* by R factors and transfer factors

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SUMMARY

A total of 2716 R factors and transfer factors isolated from *Escherichia coli* and salmonellas of human and animal origin were studied for their phage-restrictive effects in *Salmonella typhimurium* phage type 36. All of 1402 wild *fi*$^+$ factors were non-restricting. The F factor of *E. coli* K12 was unique among the F-like factors tested in that it inhibited lysis of type 36 by one typing phage. In contrast, eleven distinct changes in the phage type of 36 were produced by *fi*$^-$ I-like factors. I-like plasmids can thus be subdivided by this method.

I-like R factors and transfer factors from human and animal enterobacteria were categorized by their phage-restrictive effects in type 36. Factors resembling Δ in this respect predominated among *fi*$^-$ I-like factor from human *E. coli* and *S. typhimurium* and from porcine *E. coli*. Δ-like and ColI-like *fi*$^-$ factors were equally distributed in bovine *S. typhimurium*. ColI-like factors were commonest in bovine and avian *E. coli*.

INTRODUCTION

Restriction of bacteriophage lysis by R factors was first demonstrated by Watanabe *et al.* (1964), when *fi*$^-$ R factors introduced into *Escherichia coli* K12 were shown to inhibit the multiplication of externally infecting λ and T1 phages. Anderson & Lewis (1965*b*) and Anderson (1966) found that some *fi*$^-$ R factors restricted lysis by the *Salmonella typhimurium*, *S. paratyphi B* and *S. typhi* typing phages, reducing the sensitivity of the host strains in characteristic fashion, and thereby altering the 'phage type' of the salmonellas concerned. Carriage of the Δ transfer factor and its derivative R factor T-Δ, for example, reduced the sensitivity of *S. typhimurium* phage type 36 (= type 36), which is sensitive to all of the 30 *S. typhimurium* typing phages, to type 6, which is sensitive to only six of the phages.

These results led to the suggestion that the subdivision of R factors and transfer factors into *fi*$^-$ and *fi*$^+$ classes could be supplemented by determination of their bacteriophage restriction in *E. coli* K12 and in standard salmonella hosts (Anderson, 1966). This paper describes the effects of R factors and transfer factors on the sensitivity of type 36 to the *S. typhimurium* typing phages. The material examined

Table 1. *Laboratory strains used as recipients*

ERL No.	Description	Drug resistance*	Designation
19R689	*S. typhimurium* phage type 36	Sensitive	Type 36
14R525	*E. coli* K12F$^-$*lac*$^+$Nx^r†	Nx	K12
13R140	*E. coli* K12F$^-$*lac*$^+$	SSu‡	K12 SSu
16R99	*E. coli* K12F$^-$lac$^+$	K‡	K12K
22R721	*S. typhimurium* phage type 36	SSu	Type 36 SSu
4R914	*S. typhimurium* phage type 36	K	Type 36 K

* K, Neomycin-kanamycin; S, streptomycin; Su, sulphonamides.
† Nalidixic acid-resistant mutant.
‡ Resistance determinants only.

was isolated from human and animal enterobacteria during the 3 year period 1969–72. Detailed descriptions of this survey, and of various aspects of it, are in preparation.

MATERIALS AND METHODS

Transfer of R factors and transfer factors to type 36

The conjugation techniques used were those of Anderson & Lewis (1965*a*, *b*).

Transfer from E. coli

Wild, drug-resistant strains of *E. coli* were crossed with strain 19R689, a nalidixic acid-resistant mutant of *S. typhimurium* type 36. After about 16 hr., mating mixtures were plated out on MacConkey agar plates containing the appropriate drug plus 40 μg/ml. of nalidixic acid to eliminate the drug-resistant donor strains. Transfer factors in drug-sensitive *E. coli* were detected by their ability to mobilize 'standard' streptomycin-sulphonamide (SSu) and neomycin-kanamycin (K) resistance determinants by the triparental cross for determinant mobilization (Anderson, 1965), using *S. typhimurium* type 36 (19R689) as the final recipient. The SSu determinant is most easily mobilized by I-like transfer factors, whereas the K determinant is best mobilized by F-like transfer factors (Anderson, 1968).

Transfer from salmonellas

R factors from drug-resistant salmonellas were first transferred to strain 14R525, a nalidixic acid-resistant mutant of *E. coli* K12F$^-$lac$^+$ (= K12). They were then transferred from 14R525 to type 36, counter-selecting against the K12 donor with colicin E2 (Anderson & Lewis, 1965*a*, *b*). Transfer factors in drug-sensitive salmonellas were again detected by the triparental cross for mobilization of the SSu and K determinants, using K12 as the final recipient.

The characters of the laboratory strains of *S. typhimurium* and K12 used in these investigations are summarized in Table 1.

Phage-typing

Recipient strains of type 36 were phage-typed by the methods of Callow (1959) and Anderson (1964, and in preparation).

Bacterial strains

Type strains	Source	ERL no.	Drug resistance	Transfer factor type‡
Type 36	Bovine	8M677	Sensitive	.
Type 125	Unknown	4M2563	Sensitive	*fi*⁻ I-like (ColI)
Type 6	Human	M736	Sensitive	*fi*⁻ I-like (Δ)
Type 29	Human	M3878	Sensitive	*fi*⁻ I-like (Δ)
Type 29	Bovine	RT1	ASSuTFu	*fi*⁻ I-like (Δ)

Origin of R factors and transfer factors	Source	ERL no.	Drug resistance§	Resistance transferred to type 36
E. coli	Human	27R301	CSSu (ColI)	CSSu (ColI)
S. typhimurium type 6	Human	12R1373	T	T
E. coli	Human	27R379	AKS	AKS
E. coli	Human	27R867	ASSuT	AS
E. coli	Human	27R696	ASSuT	ASSu
E. coli	Human	27R1	ASSu	A
E. coli	Human	27R315	ACKSSuT	ACKSSuT
S. paratyphi B (Beccles)	Human	27R39	AT	AT
E. coli	Human	29R59	ACT	ACT
S. paratyphi B (Taunton var. 1)	Human	23R8	T	T
S. typhimurium type 145	Human	24R816	Sensitive, TF⁺‖	TF only
S. typhimurium type 29	¶		A	A-Δ
F factor (F-*lac*)	*E. coli* K12		Sensitive, TF⁺	TF only

Table 2. *Phage restriction in* Salmonella typhimurium *phage type* 36 *by R factors and transfer factors*

A. Type strains

Reactions with routine test dilutions of the typing phages*																														Phage type† (Callow, 1959; Anderson, 1964, and in preparation)
1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
SCL	CL	CL	CL	CL	CL	CL	CL	CL	CL	SCL	CL	CL	CL	CL	SCL	CL	CL	CL	CL	CL	CL	CL	CL	SCL	CL	OL	CL	SCL	OL	36
+ + +	SCL	+ + +	SCL	CL	+ + +	SCL	SCL	SCL	OL	–	–	CL	CL	OL	+ + +	CL	OL	SCL	SCL	SCL	CL	SCL	SCL	SCL	CL	CL	CL	SCL	OL	125
–	–	–	–	+	CL	–	–	–	–	–	–	–	–	CL	+	–	–	–	–	–	–	–	–	–	SCL	–	OL	–	SCL	6
–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+ + +	–	CL	–	–	29
–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+ + +	–	CL	–	–	29

B. Type 36 carrying R factors and transfer factors from wild strains

Transfer factor type	Reactions with routine test dilutions of the typing phages																													
	1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
fi⁻ I-like	+ + +	CL	SCL	CL	CL	OL	OL	CL	SCL	OL	–	–	CL	OL	OL	+ + +	CL	CL	CL	CL	OL	CL	CL	CL	SCL	CL	OL	CL	CL	OL	125
fi⁻ I-like	–	–	–	–	–	SCL	–	–	–	–	–	–	–	–	SCL	–	–	–	–	–	–	–	–	–	–	+	–	+ + +	–	+	6
fi⁻ I-like	–	–	–	–	–	SCL	–	–	–	–	–	–	–	–	SCL	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	145
fi⁻ I-like	–	+	–	–	SCL	+	+	–	–	+	–	–	CL	CL	+ + +	+	–	+ + +	+	+	+	+	+	+	+	CL	SCL	CL	+	OL	NC 1
fi⁻ I-like	–	–	–	–	–	–	–	CL	–	+ + +	SCL	CL	–	+ + +	OL	+	CL	+ + +	–	–	+ +	+ + +	–	SCL	–	+	OL	SCL	+ +	+	NC 2
fi⁻ I-like	–	–	–	–	–	–	–	+ + +	–	OL	+	+ +	OL	–	+ +	–	+ + +	–	–	–	–	–	–	–	–	SCL	+ + +	OL	–	OL	85
fi⁻ I-like	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CL	–	–	–	–	–	–	–	–	–	–	21
fi⁻ I-like	–	–	–	–	+	SCL	–	–	–	–	CL	CL	+ + +	+	SCL	–	–	+	–	CL	–	–	+	–	–	–	+	+ + +	–	+	NC 3
fi⁻ I-like	–	–	+	+	+ +	OL	–	–	–	+	SCL	CL	OL	+ + +	OL	+	–	+	–	CL	–	–	+	–	–	+ +	+	+ + +	–	SCL	NC 4
fi⁻ I-like	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	U
fi⁻ I-like	+	+ + +	+ +	–	–	–	+ + +	–	SCL	SCL	–	–	CL	–	CL	+	–	SCL	SCL	SCL	+ +	SCL	SCL	SCL	+	+	+	CL	+ +	+ + +	NC 5
fi⁻ I-like	+	+	+	+ + +	+ + +	SCL	+ + +	SCL	+	+ + +	–	–	CL	SCL	CL	+ +	SCL	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+	CL	CL	CL	+	OL	NC 6
F factor	SCL	CL	CL	CL	CL	CL	–	CL	CL	CL	SCL	CL	CL	CL	CL	SCL	CL	CL	CL	CL	CL	CL	CL	CL	SCL	CL	OL	CL	SCL	OL	NC 7

* Reactions with typing phages: CL, confluent lysis; SCL, semi-confluent lysis, OL, confluent lysis with a heavy central opacity due to secondary growth; + to + + +, increasing number of discrete plaques.
† U = does not react with typing phages; NC (1–7) = react with typing phages but do not conform to a designated type.
‡ *fi* = fertility inhibition; I-like (see Moynell, Meynell & Datta, 1968); (ColI) = colicin I-producing; (Δ⁺) = carrying the Δ transfer factor (Anderson & Lewis, 1965*b*).
§ Resistance symbols: A, ampicillin; C, chloramphenicol; K, neomycin-kanamycin; S, streptomycin; Su, sulphonamides; T, tetracyclines; Fu, furazolidone.
‖ TF⁺ = carrying transfer factor only.
¶ Artificially made in the laboratory (see Anderson, 1969).

Determination of the fi *character and* I *specificity of* R *factors and transfer factors*

The *fi* character of R factors and transfer factors was examined by the method of Pitton & Anderson (1970). The I-like character was determined by testing the ability of the host strains to support growth of phage If1 (Meynell & Lawn, 1968).

RESULTS

Phage restriction in S. typhimurium *phage type* 36 *by* R *factors and transfer factors*

Thirteen different patterns of typing phage restriction were produced by various R factors and transfer factors in type 36 of *S. typhimurium*. These patterns, and the control reactions of types 36, 125, 6, and 29 of *S. typhimurium* with the thirty *S. typhimurium* typing phages, are summarized in Table 2.

Ten R factors and one transfer factor producing the phage restriction patterns in *S. typhimurium*, shown in Table 2, were isolated from wild strains of *S. typhimurium*, *S. paratyphi B* and *E. coli* of human origin. The F factor of *E. coli* K12F$^+$, and the A-Δ R factor artificially produced in the laboratory (Anderson, 1969), also caused phage restriction. With the exception of the F factor, all phage-restricting factors were *fi*$^-$ and I-like.

Five patterns of typing phage restriction, produced by R factors from the *E. coli* strain 27R301, the *S. typhimurium* type 6 strain 12R1373, and the *E. coli* strains 27R379, 27R1 and 27R315, corresponded to known phage types of *S. typhimurium*. These were types 125, 6, 145, 85 and 21 respectively. The phage restriction in *S. typhimurium* resulting from introduction of R factors from *E. coli* 27R867, 27R696, 29R59, and from *S. paratyphi B* strain 27R39, did not correspond to known phage types of *S. typhimurium*. Nor did those produced by the A-Δ factor, by the factor from *S. typhimurium* strain 24R816, and by the F factor of K12. These seven restriction patterns were designated 'non-conforming' (NC) 1–7. Finally, one R factor, isolated from *S. paratyphi B* strain 23R8, restricted all the *S. typhimurium* typing phages. Type 36 carrying this R factor was therefore designated untypable (U).

The drug-sensitive type strains of *S. typhimurium* types 125, 6, 29 and 145, proved to be carrying *fi*$^-$ I-like transfer factors. The factor from 4M2563, the type strain of 125, also carried the genetic determinant for the production of colicin I (ColI$^+$). M736, the type strain of type 6, isolated in 1945, carries a transfer factor yielding restriction identical with that of Δ, which converts type 36 into type 6 (Anderson & Lewis, 1965*b*). The R factor from the wild *S. typhimurium* type 6 strain 12R1373, isolated in 1971, was also Δ-like. The R factor from the wild strain of *E. coli* 27R301 restricted phages 12 and 13 of the *S. typhimurium* typing scheme, thereby producing type 125; it was also ColI$^+$. The *fi*$^-$ I-like factor from 5M4750, the type strain of type 145, isolated in 1965, converted type 36 to type 145, as did the R factor from the wild *E. coli* strain 27R379. Although the differences between types 6 and 145 are small, they are nevertheless constant.

Table 3. *Source of* fi⁻ *I-like R factors and transfer factors from human strains of enterobacteria*, 1969–1972

Phage type produced in type 36	Source of factor					
	E. coli	*S. typhimurium*	*S. enteritidis*	*S. paratyphi B*	Total	%
125 ColI⁺	8 (2)	2 (2)	9 (3)	0	19 (7)	3·8
125 Col⁻	15 (5)	2 (2)	0	0	17 (7)	3·4
Total	23 (7)	4 (4)	9 (3)	0	36 (14)	7·1
6	104 (11)	141 (5)	0	2 (1)	247 (17)	48·8
145	13 (2)	2 (1)	0	0	15 (3)	3·0
NC 1	12 (1)	0	0	0	12 (1)	2·4
NC 2	12 (1)	1 (1)	0	0	13 (2)	2·6
85	1 (1)	0	0	0	1 (1)	0·2
21	0	1 (1)	0	0	1 (1)	0·2
NC 3	0	0	0	4 (2)	4 (2)	0·8
NC 4	8 (1)	0	0	0	8 (1)	1·6
U	11 (3)	1 (1)	0	1 (1)	13 (5)	2·6
NC 5	0	1 (1)	0	0	1 (1)	0·2
fi⁻nr I-like	86 (10)	37 (3)	10 (5)	22 (3)	155 (21)	30·6
Total	270 (37)	188 (17)	19 (8)	29 (7)	506 (69)	
% of overall total	53·4	37·1	3·8	5·7		

SYMBOLS. See Table 2.
ColI⁺ = transfer factors with I, Ia or Ib colicinogeny determinants.
Col⁻ = non-colicinogenic.
fi⁻nr = fertility inhibition minus, non-restricting for *S. typhimurium* typing phages.
All of 69 factors tested, shown in parentheses, were I-like.

Sources of fi⁻ *I-like R factors and transfer factors from human enterobacterial strains*

A total of 1060 R factors and transfer factors from human strains of *E. coli*, *S. typhimurium*, *S. paratyphi B* and *S. enteritidis* were examined for restriction of the *S. typhimurium* typing phages. These transfer systems will be described in detail in later articles. Of 554 factors that were *fi*⁺ and non-restricting, 503 (90·8 %) were R⁺.* The remaining 506 factors were *fi*⁻ and included 463 (91·5 %) R⁺ lines. All of 69 *fi*⁻ factors (62 = 90 % R⁺) investigated promoted multiplication of the If1 phage, and were therefore I-like.

The enterobacterial sources of *fi*⁻ factors, both restricting and non-restricting for *S. typhimurium* typing phages, are shown in Table 3, and the proportion carrying resistance in Table 4.

Of 506 of these factors examined, 270 (234 = 86·7 % R⁺) were isolated from wild *E. coli* strains, 188 (181 = 96·3 % R⁺) from *S. typhimurium*, 19 R factors from *S. enteritidis* and 29 R factors from *S. paratyphi B*.

155 factors (134 = 86 % R⁺) were *fi*⁻ and non-restricting (= *fi*⁻nr) for the *S. typhimurium* typing phages; all of 21 (18 R⁺) of this group examined were

* The symbol R⁺ is used here to indicate transfer factors associated with R determinants; that is, R factors.

Table 4. fi⁻ *I-like R factors from human strains of enterobacteria*, 1969–1972

Phage type produced in type 36	Source of factor: *E. coli*	*S. typhimurium*	*S. enteritidis*	*S. paratyphi B*	Total
125 ColI⁺	8	2	9	0	19
125 Col⁻	15 (12)	2	0	0	17 (14)
Total	23 (20)	4	9	0	36 (33)
6	104 (92)	141 (134)	0	2	247 (228)
145	13	2	0	0	15
NC 1	12	0	0	0	12
NC 2	12	1	0	0	13
85	1	0	0	0	1
21	0	1	0	0	1
NC 3	0	0	0	4	4
NC 4	8	0	0	0	8
U	11	1	0	1	13
NC 5	0	1	0	0	1
fi⁻nr I-like	86 (65)	37	10	22	155 (134)
Total	270 (234 = 86·7 %)	188 (181 = 96·3 %)	19	29	506 (463 = 91·5 %)

Symbols. See Tables 2 and 3.

When all transfer systems were R⁺, the figure alone is given. When some transfer systems were R⁻, figures in parentheses indicate the numbers that were R⁺.

Percentage resistance is shown at foot of Table.

I-like. Factors producing the Δ type of phage restriction (= Δ-like) were predominant amongst the *fi*⁻ factors tested; 247 (228 = 92·3 % R⁺) of 506 factors (48·8 %) converted type 36 to type 6; all of 17 (15 R⁺) Δ-like *fi*⁻ factors examined were I-like.

Surprisingly few R factors isolated from human strains carried the determinant for colicin I production; only 19 (3·8 %) were ColI⁺ and converted type 36 to type 125. However, a further 17 non-colicinogenic factors (14 R⁺) restricted only phages 12 and 13 of the *S. typhimurium* typing scheme. This restriction yields type 125 and is characteristic of most ColI factors. All of 7 R factors of this class tested were I-like; their transfer factors, which produced the phage restriction, were evidently similar to that of ColI factors.

Nine phage types were produced by the remaining 68 *fi*⁻ R factors from human enterobacterial strains. Only the types designated NC 6 and NC 7 (see Table 2), which had arisen from infection of type 36 with the 'synthetic' R factor A-Δ and the F factor respectively, were not produced by factors from wild human strains. After the Δ-like and ColI-like factors, the commonest phage-restricting factor found was that which converted type 36 to type 145. Fifteen R factors gave rise to this phage type, and all of 3 tested were I-like.

Factors from human E. coli. *Total* 270. 234R$^+$ (86·7 %)

Eight different patterns of phage restriction of *S. typhimurium* type 36 were produced by the 270 *fi*$^-$ factors from human *E. coli*. 104 *fi*$^-$ factors (92 = 88·5 % R$^+$) were Δ-like, 86 (65 = 75·6 % R$^+$) were non-restricting for the typing phages, and 23 (20 R$^+$) gave rise to type 125. However, 57 R factors yielded phage types other than 6 and 125. Eleven R factors from *E. coli* completely inhibited lysis by all the *S. typhimurium* typing phages.

Factors from human S. typhimurium. *Total* 188. 181 R$^+$ (96·3 %)

The majority of *fi*$^-$ factors from human strains were Δ-like: 141 (74·5 %) of 188 strains from 5 phage types of *S. typhimurium* carried this class of factor; 134 (95 %) of the Δ-like factors were R$^+$. Thirty-seven of the 188 strains (19·7 %) carried *fi*$^-$ I-like R factors which did not restrict the typing phages, and 10 carried R factors giving rise to 6 other distinctive patterns of phage restriction: 21, 125, 145, NC 2, NC 5 and U. Only 4 R factors, 2 of which were ColI$^+$, produced type 125.

Factors from human S. enteritidis. *Total* 19. *All* R$^+$

Nineteen *fi*$^-$ R factors were isolated from drug-resistant *S. enteritidis* strains. Ten were non-restricting for typing phages, and all of 5 tested were I-like. Drug resistance transfer in the remaining 9 strains was mediated by a ColI$^+$ factor, which converted type 36 of *S. typhimurium* into type 125.

Factors from human S. paratyphi *B*. *Total* 29. *All R*$^+$

Twenty-two of 29 resistance factors from *S. paratyphi B* strains did not cause restriction in type 36; all of 7 factors of this group examined were I-like. However, 7 *fi*$^-$ R factors, of which all of 4 examined were I-like, produced phage restriction in type 36: two R factors were Δ-like, 4 gave rise to the NC 3 phage type, and 1 restricted lysis by all the *S. typhimurium* typing phages.

Sources of fi$^-$ *I-like R factors and transfer factors from animal enterobacterial strains*

A total of 1656 *fi*$^-$ and *fi*$^+$ factors from bovine, porcine and avian strains of *E. coli* and *S. typhimurium* were examined. All avian strains were isolated from broiler fowls. 848 factors (51·2 %), of which 566 were from bovine, 198 from porcine and 84 from avian enterobacteria, were *fi*$^+$; 512 *fi*$^+$ factors (90·5 %) from bovine strains and all *fi*$^+$ factors from porcine and avian enterobacteria were R$^+$. Of 126 *fi*$^+$ R factors tested, 86 bovine, 29 porcine and 11 avian in origin, all were non-restricting (= *fi*$^+$nr) for the *S. typhimurium* typing phages.

The sources of the *fi*$^-$ I-like factors from the bovine, porcine, and avian enterobacteria, and the phage restriction produced by these factors in type 36, are presented in Table 5; the proportion carrying resistance in Table 6.

Of 808 *fi*$^-$ factors (750 = 92·8 % R$^+$) tested for phage restriction, 271 (240 = 88·6 % R$^+$) were from strains of bovine *E. coli* and 61, all R$^+$, from bovine *S. typhimurium*; 211 *fi*$^-$ factors (201 = 95·3 % R$^+$) came from porcine *E. coli*; 2, both R$^+$,

Table 5. *Source of* fi⁻ *I-like R factors and transfer factors from animal strains of enterobacteria*, 1969–1972

Phage type produced in type 36	Source of factor: Bovine *S. typhimurium*	Bovine *E. coli*	Porcine *S. typhimurium*	Porcine *E. coli*	Avian *S. typhimurium*	Avian *E. coli*	Total
125 ColI⁺	30 (3)	127 (6)	0	21 (2)	3 (3)	98 (4)	279 (18)
125 Col⁻	0	82 (2)	0	1 (1)	0	102 (16)	185 (19)
Total	30 (3)	209 (8)	0	22 (3)	3 (3)	200 (20)	464 (37)
6	30 (5)	3 (3)	2 (2)	157 (3)	0	47 (5)	239 (18)
NC 1	0	2 (1)	0	0	0	0	2 (1)
U	0	2 (1)	0	0	0	0	2 (1)
fi⁻nr I-like	1 (1)	55 (6)	0	32 (1)	0	13 (2)	101 (10)
Total	61 (9)	271 (19)	2 (2)	211 (7)	3 (3)	260 (27)	808 (67)
% of overall total	7·5	33·5	0·2	26·1	0·4	32·2	

SYMBOLS. See Tables 2 and 3.
All of 67 factors tested, shown in parentheses, were I-like.

Table 6. fi⁻ *I-like R factors from animal strains of enterobacteria*, 1969–1972

Phage type produced in type 36	Source of factor: Bovine *S. typhimurium*	Bovine *E. coli*	Porcine *S. typhimurium*	Porcine *E. coli*	Avian *S. typhimurium*	Avian *E. coli*	Total
125 ColI⁺	30	127 (112)	0	21	3	98 (90)	279 (256)
125 Col⁻	0	82 (72)	0	1	0	102 (93)	185 (166)
Total	30	209 (184)	0	22	3	200 (183)	464 (422)
6	30	3	2	157 (150)	0	47	239 (232)
NC 1	0	2	0	0	0	0	2
U	0	2	0	0	0	0	2
fi⁻nr I-like	1	55 (49)	0	32 (29)	0	13	101 (92)
Total	61	271 (240 = 88·6 %)	2	211 (201 = 95·3 %)	3	260 (243 = 93·5 %)	808 (750 = 92·8 %)

SYMBOLS: see Tables 2 and 3.
When all transfer systems were R⁺, the figure alone is given. When some transfer systems were R⁻, figures in parentheses indicate the numbers that were R⁺. Percentage resistance is shown at foot of table.

from porcine *S. typhimurium*; 260 (243 = 93·5 % R⁺) were from avian *E. coli*, and 3, all R⁺, from avian *S. typhimurium*.

A hundred and one *fi*⁻ factors (92 = 91·1 % R⁺) were non-restricting for the *S. typhimurium* typing phages. The predominant phage-restricting effect was to change type 36 into type 125; 464 factors (422 = 90·9 % R⁺), 279 of which were ColI⁺, and 256 (91·8 %) of these R⁺, gave rise to this phage type. 239 factors

(232 = 97·1 % R^+) were Δ-like, converting type 36 into type 6. All of 18 factors (16 R^+) of this class tested were I-like. Only 4 R factors produced other restriction in type 36: 2 completely inhibited lysis (U), and 2 gave rise to the NC 1 type (see Table 2).

Factors from bovine enterobacteria. Total 332. 321 R^+ (96·7 %)

S. typhimurium. *Total* 61. *All* R^+. Sixty-one *fi*$^-$ R factors from bovine *S. typhimurium* strains were examined; 30 were ColI$^+$ and converted type 36 to type 125; 30 were Δ-like and changed type 36 into type 6. Only one *fi*$^-$ I-like factor was non-restricting for the *S. typhimurium* typing phages.

E. coli. *Total* 271. 240 R^+ (88·6 %). Restriction in type 36 with *fi*$^-$ factors from bovine *E. coli* was rather different from that with factors from the *S. typhimurium* strains. Whereas 30 of 61 factors (49·2 %) from bovine *S. typhimurium* were Δ-like, only 3 R factors of 271 *fi*$^-$ factors (1·1 %) from *E. coli* belonged to that category. The predominant type of factor from bovine *E. coli* was that which converted type 36 into type 125; 209 (184 = 88·1 % R^+) of 271 factors (77·1 %) gave rise to this phage type, and 127 (60·8 %) were ColI$^+$. Eighty-eight of the 127 ColI$^+$ factors (69·2 %) carried a K determinant. The transfer factor with which the ColI determinant is usually associated is evidently common in bovine *E. coli*. The two *fi*$^-$ R factors resulting in the NC 1 type, and the two blocking lysis by all the typing phages, originated in bovine *E. coli*.

Factors from porcine enterobacteria. Total 213. 203 R^+ (95·3 %)

S. typhimurium. *Total* 2. *Both* R^+. Only three strains of porcine *S. typhimurium* were examined for R factors or transfer factors in this survey. Two strains, both belonging to type 6, carried Δ-like R factors; the third strain tested, type 12a, was drug-sensitive and did not carry a transfer factor.

E. coli. *Total* 211. 201 R^+ (95·3 %). In contrast to the factors from bovine *E. coli*, 157 (150 = 95·5 % R^+) of 211 *fi*$^-$ factors (74·4 %) from porcine *E. coli* were Δ-like. Twenty-two *fi*$^-$ R factors, of which 21 were ColI$^+$, yielded type 125, and 32 (29 R^+) were non-restricting for the typing phages. No other restriction patterns were encountered.

Factors from avian enterobacteria. Total 263. 246 R^+ (93·5 %)

S. typhimurium. *Total* 3. *All* R^+. Only 3 strains of avian *S. typhimurium* carrying *fi*$^-$ R factors were examined in this survey. All 3 factors from these strains were ColI$^+$, and changed type 36 into type 125.

E. coli. *Total* 260. 243 R^+ (93·5 %). Two hundred (183 = 91·5 % R^+) of 260 *fi*$^-$ factors (77·0 %) from avian *E. coli* yielded type 125 when introduced into type 36; of these, 98 (90 = 91·8 % R^+) were ColI$^+$, and 102 (93 = 91·2 % R^+) were non-colicinogenic. As can be expected, all of 20 factors (16 R^+) of this class examined were I-like. Δ-like R factors were present in 47 *E. coli*, and *fi*$^-$ R factors non-restricting for typing phages in a further 13 strains.

Table 7. *Main categories and origins of R factors and transfer factors*

Source		Total no. of factors	Type of factor: No.			Type of factor: Percentage		
Species	Organism		Δ-like	ColI-like	*fi*⁻nr	Δ-like	ColI-like	*fi*⁻nr
Human	*E. coli*	270	104	23	86	38·5	8·5	31·9
Human	*S. typhimurium*	188	141	4	37	75·0	2·1	19·7
Bovine	*E. coli*	271	3	209	55	1·2	77·1	20·3
Bovine	*S. typhimurium*	61	30	30	1	49·2	49·2	1·6
Porcine	*E. coli*	211	157	22	32	74·4	10·4	15·2
Avian	*E. coli*	260	47	200	13	18·1	76·9	5·0

DISCUSSION

Of the 2716 transfer systems from human and animal enterobacteria studied, 1402 (51·6 %) were *fi*⁺ and caused no restriction in type 36 of *S. typhimurium*. The remaining 1314 factors were *fi*⁻, and all of 136 factors of this group tested were I-like. With the exception of the F-factor of *E. coli* K12, all phage-restricting factors were *fi*⁻ and I-like. Restriction patterns in type 36 may therefore be added to the criteria available for the characterization, and thus the classification, of transfer systems (Anderson, 1966).

Eleven distinct changes in *S. typhimurium* type 36 were produced by infection with *fi*⁻ I-like factors from wild *E. coli* and salmonellas. The F-factor was unique among F-like factors tested in that it restricted one of the *S. typhimurium* typing phages. However, all other F-like factors tested were compatible with the F-factor of K12, an indication that they are different from F.

Three *fi*⁻ factors, Δ, ColI and the factor from 5M4750, determined the phage types of the *S. typhimurium* strains in which they were first encountered. Although all are 'I-like', their phage-restricting effects on strains of *S. typhimurium* are quite different. The Δ-like factors, the prototype of which was described by Anderson & Lewis (1965*b*), inhibit lysis by 24 of the 30 *S. typhimurium* typing phages, to produce phage type 6. The ColI-like factors inhibit lysis by only two typing phages, to give rise to type 125 (Anderson, 1966), and the factor from *S. typhimurium* 5M4750 inhibits lysis by 27 typing phages, to produce type 145.

The three main groups of factors, and their origins, are shown in Table 7.

Among the *fi*⁻ I-like factors the Δ-like group predominated in human *E. coli* and *S. typhimurium*: 104 out of 270 factors (38·5 %) from human *E. coli* belonged to this category. In human *S. typhimurium* infection the preponderance of Δ-like factors was more marked: 141 of 188 *fi*⁻ factors (75 %) were of this type. All of 118 strains of phage type 6, of which 111 were R⁺, carried this class of factor; a significant number when it is recalled that the phage-restricting effect of the Δ group of plasmids on *S. typhimurium* type 36 is to change it into type 6, and that the drug-sensitive type strain of type 6, which was identified almost 30 years ago, carries a Δ-like factor. It is thus probable that the phage type-determining agent in all strains of type 6 is a transfer factor of the Δ group. This has proved to be

true in every strain of type 6 so far examined. Transfer of R factors of this group would also be expected to occur in other types of *S. typhimurium*, and this was found, for example, in the 23 strains belonging to types other than 6 among the 141 Δ-carrying lines of *S. typhimurium* identified.

Bovine *S. typhimurium* yielded 23 type 6 strains, all of which were drug-resistant, and seven strains belonging to other phage types also carried Δ-like factors.

The most important strain of *S. typhimurium* in man and bovines which carries a Δ transfer system is that of phage type 29, which caused a prolonged epidemic of bovine and human infection between 1964 and 1970. It has been postulated elsewhere that this outbreak was caused by a single line, in fact a clone, of type 29 (Anderson, 1968, 1969, 1971). It so happened that in the original group of drug-resistant *S. typhimurium* cultures isolated from November 1964 onwards on a farm in Devon, 54 belonged to phage type 29 and 35 to phage type 44. A recent scrutiny of these cultures has shown that the type 44 cultures, all of which are multi-resistant, carry a transfer factor of the ColI-like group – that is, a factor which converts type 36 into type 125. All these cultures are ColI$^-$.

Type 44 had a long history of bovine association before the appearance of transferable resistance, and its pattern of typing phage sensitivity is unchanged by the introduction of a ColI-like transfer factor. Type 29, in contrast, had been uncommon before the widespread outbreak it caused in bovines and man. The first strain of this type identified in that outbreak has been intensively studied in the Enteric Reference Laboratory, and is designated RT1 (Anderson & Lewis, 1965*a*, *b*; Anderson, 1966, 1968, 1969). It was the source of Δ, the prototype of the Δ-like group of transfer factors. Δ is incompatible with the ColI-like factors. Introduction of a ColI factor into RT1 resulted in displacement of the Δ factor (the resistance determinants were unaffected), and the strain simultaneously changed into phage type 44 (E. S. Anderson & H. R. Smith, unpublished observations). This revealed that the extensive type 29 outbreak in bovines and man had been caused by one strain of a type that had long been associated with bovines, and not by a newly introduced type of *S. typhimurium*. We do not yet know why the Δ-carrying line should have persisted and spread while that carrying the ColI-like (but Col$^-$) transfer factor disappeared. But we now know that all strains of type 29, whether or not they are drug-resistant, carry a Δ-like factor. By good fortune, *S. typhimurium* 4M5235, the drug-sensitive 'type 29' line on which the original experiments were carried out to examine its ability to accept transferable resistance descended from RT1 (Anderson & Lewis, 1965*a*, *b*), was later found to belong, in fact, to type 16, which does not carry a transfer factor, although its sensitivity to the typing phages closely resembles that of type 29. Had the sensitive recipient really belonged to type 29, difficulties would have occurred because a Δ-like transfer factor would already have been present, so that the resistance transfer findings could have been confused.

In the study reported here, factors yielding phage type 125 in *S. typhimurium* were of two types; those carrying a colicin I determinant (= ColI$^+$), and those which were Col$^-$; each of these types showed R$^+$ and R$^-$ representatives. Colicin I determinants alone are non-transferring, and require association with a transfer

factor to become transferable. Why they should be associated particularly with this class of transfer factor is not clear, since their transfer is easily mediated by Δ. Perhaps the '125' type of transfer factor has the greatest long-term stable linkage with ColI determinants.

Relatively few factors from human salmonellas gave rise to type 125, but factors of this class were particularly common in bovine and avian *E. coli*, and to a lesser extent in porcine *E. coli*. Animal *E. coli* probably constitute a reservoir of this type of factor, which, under suitable conditions, can enhance the spread of drug resistance to pathogenic bacteria. A relatively common R factor in *S. typhimurium* and *E. coli* is KColI, in which both the neomycin–kanamycin R determinant and a ColI determinant are linked to the same transfer factor. The use of neomycin or kanamycin against infection with *S. typhimurium* carrying KColI therefore selects for colicinogeny as well as kanamycin resistance. The spread of drug resistance by a ColI factor has recently been particularly evident in enterobacteria from broiler fowls (E. S. Anderson & L. G. Savoy, in preparation).

Although many *fi*⁻ I-like R factors and transfer factors could be distinguished by their phage restriction, a considerable proportion were non-restricting for *S. typhimurium* typing phages, and therefore could not be differentiated further on this basis. Supplementary subdivision of Δ-like factors, and of factors giving rise to type 125, would be advantageous. Differences between the phage-restricting effects of Δ and those of a modified form of Δ, designated Δm, were apparent in Vi-type A of *S. typhi* (Anderson, 1966). This host, or others, may therefore be useful for the subdivision of R factors and transfer factors that produce identical phage restriction, or lack of it, in *S. typhimurium*.

Phage restriction in *E. coli* and salmonellas may also be useful in the classification of F-like transfer systems, although the 680 tested in this survey produced no *S. typhimurium* phage restriction. However, the F factor itself has been shown here to restrict one of the *S. typhimurium* typing phages, and we have an F-like R factor that also restricts lysis by these phages in a manner different from that of F.

From the taxonomic point of view, the designation of transfer systems as I-like (Meynell, Meynell & Datta, 1968) is unfortunate. The letter I was introduced because the respective sex fimbriae ('I' fimbriae) were first observed in a strain carrying the classical ColI-P9 plasmid (Fredericq, 1956). The If1 phage, for which I fimbriae are the receptor, was isolated by Meynell & Lawn (1968), on a derivative of a strain (*S. typhimurium* ERL ref. 4M91) carrying the ColI⁺-kanamycin R factor R144, and on a derivative of a line (*S. typhimurium* ERL ref. 3M2318) carrying the Col⁻R⁺ factor R64. R64 is a Δ-like factor. The transfer of ColI is mediated by a transfer factor (TF) with which the genetic determinant of I colicinogeny is covalently linked. We have found that the 'ColI' system can exist in the following states: ColI⁺TF⁺, ColI⁺TF⁻ and ColI⁻TF⁺. ColI⁺TF⁻ strains produce colicin I but do not support the growth of phage If1 because they lack 'I' fimbriae. ColI⁻TF⁺ strains, in contrast, are non-colicinogenic, but synthesize 'I' fimbriae and therefore support the growth of phage If1; they produce the 125 type of restriction in type 36 of *S. typhimurium*. Since the 'I' designation of the transfer factor they carry was derived from that of a ColI⁺TF⁺ R factor, it is misleading to attach the I symbol

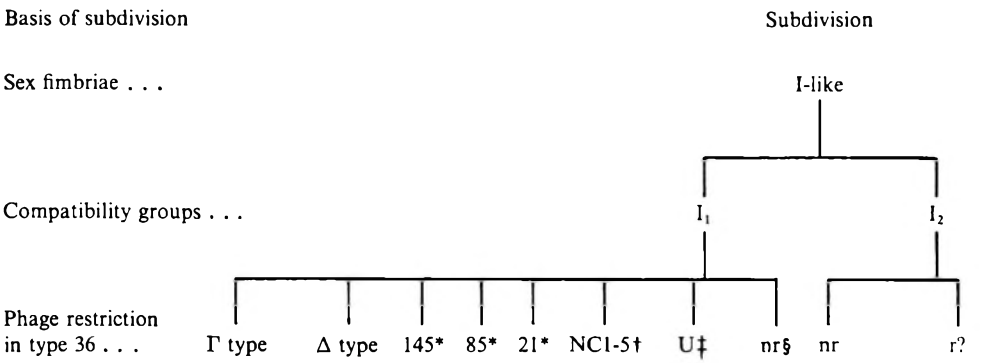

Fig. 1. Subdivision of I-like plasmids. * The phage type numbers indicate the restriction pattern and the provisional plasmid type designation. † NC1–5: specific restriction patterns, each indicating a distinct plasmid type (see Table 2 and text). ‡ Restriction of all *S. typhimurium* typing phages and provisional plasmid type designation. § Non-restricting in *S. typhimurium*. r ? No representatives yet available.

to ColI$^-$TF$^+$ lines. Moreover, additional confusion arises from the fact that the Δ group of transfer factors also produce so-called 'I' sex fimbriae, and support growth of phage If1. They are therefore categorized as I-like, although they are rarely found in association with a ColI determinant. The same applies to all the other *fi*$^-$ I-like factors identified.

The male-specific If1 phage does not distinguish between the Δ-group and the 'ColI' group of transfer systems. Subdivision can be effected by the laborious demonstration of serological differences between the respective sex fimbriae. But the factors can be easily distinguished from each other by the clear-cut differences in phage restriction produced in type 36 of *S. typhimurium* by the Δ group of transfer factors on the one hand, and the 'ColI' group of transfer factors on the other (Anderson, 1966).

Unfortunately, the I designation has been widely accepted and it may now be difficult to abandon. Moreover, we have recently subdivided factors that stimulate synthesis of I fimbriae into groups I_1 and I_2 (Grindley, Grindley & Anderson, 1972; Grindley, Humphreys & Anderson, 1973). The I_2 group is I-like and codes for I fimbriae, but is compatible with, and therefore distinct from, Group I_1 plasmids, which are incompatible with each other. Group I_2 is at present represented by only one plasmid, TP114 (Grindley *et al.* 1972), and although it is non-restricting in *S. typhimurium*, its further characterization is as yet incomplete. Group I_1, however, is common. The Δ type of plasmids are well known, and it seems logical to designate the other common ('125') restriction type Γ(gamma), to indicate its distinctness from Δ. The other phage-restricting factors can also be provisionally regarded as representing distinct types. The non-restricting I_1 plasmids are designated 'nr', and although they are probably heterogeneous, their present designation can be retained until more is known about them. We can therefore subdivide these plasmids on the basis of sex fimbrial synthesis, compatibility and phage restriction in *S. typhimurium*, as shown in Fig. 1.

Fig. 1 indicates that a considerable heterogeneity of *fi*$^-$ I-like plasmids can be demonstrated by their patterns of phage restriction in *S. typhimurium* type 36. The

heterogeneity may be by no means fully exposed in this serotype because it may extend further than can be shown by restriction in *S. typhimurium*, or indeed by that in any host, as we have already indicated. This applies particularly to the 'U' and 'non-restricting' groups of plasmids. Moreover, it must be recalled that I-like plasmids may be *fi*$^+$ (Grindley & Anderson, 1971), and this offers further possibilities of subdivision of these factors.

The fact that most of the factors described are found in both animal and human enterobacteria suggests a common origin. And it is evident from earlier studies (Anderson & Lewis, 1965*a*, *b*; Anderson, 1968, 1969, 1971) that salmonellas carrying resistance transfer systems pass from animals to man. But the extent to which animal non-pathogens contribute to the drug-resistant enterobacterial population of the human intestine is not yet clear, although it may be substantial.

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