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Anticoagulant resistance in wild Norway rats in New York

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(*Received* 27 *July* 1972)

SUMMARY

Wild Norway rats (*Rattus norvegicus*) from several habitats were tested for their susceptibility and resistance to warfarin. Animals were fed 0·005% warfarin in ground oatmeal for periods varying from 1 to 12 days. Rats having no prior exposure to anticoagulants were shown to be susceptible, none having survived a 6-day feeding period. Resistant rats were found on two farms where anticoagulant materials had been used intensively for about 20 years.

INTRODUCTION

The anticoagulant materials proved so effective as rodenticides after their introduction in 1950 that they quickly supplanted acute rodenticides as the main materials used in the chemical control of rodents. Today, it is estimated that anticoagulants represent 95% of the United States' rodenticide market (Mampe, 1969). The world market, likewise, is thought to roughly parallel that of the United States.

The continued rodenticidal effectiveness of the anticoagulant materials has been questioned in the light of rodent resistance to them in the United Kingdom, Denmark and the United States. The first inkling of the problem came from Scotland (Boyle, 1960), where a population of *Rattus norvegicus* on a farm showed a remarkable tolerance to both warfarin and diphacinone. Additional cases of resistance in Norway rats have been uncovered in this same area of Scotland (Boyle, 1967). Meanwhile, Lund (1964) reported the discovery of anticoagulant-resistant Norway rats in the Jutland area of Denmark. Drummond & Bentley (1967) documented other anticoagulant-resistant rat populations in England and Wales and showed the tendency of resistant populations to expand radially. Ophof & Langeveld (1969) reported upon warfarin-resistant rat populations in the Netherlands and upon their subsequent extermination with the use of fluoroacetamide. The first finding of anticoagulant resistance in the United States came from Norway rats living on farms near Raleigh, North Carolina (Jackson, Spear & Wright, 1971; Jackson & Kaukeinen, 1972). The cumulative evidence suggests that wherever anticoagulants are intensively used against relatively large but persistent rat populations, resistance may be developed.

There are indications, then, that resistance may be more widespread in rat populations throughout the world than is generally recognized. Unfortunately, prompt recognition and verification of resistant populations is difficult and, until

an active effort is applied to detect resistance, the full extent of the problem will remain undisclosed.

Laboratory screening tests for anticoagulant resistance have been described (Drummond, 1966; Drummond & Wilson, 1968; WHO, 1970) and yield results that can be replicated with fair accuracy and provide comparisons with results of other workers. Using such procedures, we initiated a study of the anticoagulant susceptibility and resistance of wild Norway rats from several habitats and areas in New York.

MATERIALS AND METHODS

Wild Norway rats were captured alive in cage-traps and returned to the laboratory. They were lightly anaesthetized with carbon dioxide, weighed, measured, sexed, and caged individually. A basal diet of laboratory meal was provided. Sick, pregnant and immature animals were set aside.

After an acclimation period of 3 weeks, rats were provided with a pre-test baiting of ground oatmeal for several days until feeding was stabilized. The amount eaten daily was then measured for 2 days preceding the trial. Then, for periods ranging from 1 to 12 days, groups of rats were allowed unrestricted feeding upon ground oatmeal containing 0·005 % warfarin. The warfarin used was supplied by the Wisconsin Alumni Research Foundation and was found to be a very palatable material (Bowerman & Brooks, 1972). During and following the warfarin offering, animals were observed daily for 14 days from the start of the test for mortality, bleeding and other signs of poisoning. Dead animals were autopsied to verify anticoagulant effects. After the poisoning trial, animals were placed on a basal diet of laboratory meal. Animals were weighed immediately before the warfarin baiting and a terminal weight was obtained. The quantity of warfarin consumed was computed using the initial body weight. Animals weighing less than 150 g. were not used.

RESULTS

Susceptible rats

The purpose of the WHO testing procedure (WHO, 1970) is to measure the susceptibility of rodent populations to a given anticoagulant rodenticide. In order to detect resistant strains of rats, the normal susceptibility of populations having no known prior exposure to anticoagulants must first be established. This is then used as a measure against which suspect resistant populations are compared.

The results given in Table 1 indicate the susceptibility of two populations of Norway rats from refuse disposal sites having no known anticoagulant exposure. The population from Merrick landfill on Long Island resemble very closely data given by Drummond & Wilson (1968) for susceptible rats in England, where all rats died from 3 days exposure to 0·005 % warfarin in ground oatmeal. The second group, from Berlin Township landfill in eastern New York, survived longer feeding periods, but when the relationship between mortality and duration of feeding upon warfarin is examined (Litchfield & Wilcoxon, 1949) it indicates that

Table 1. *Mortality to warfarin-naïve wild Norway rats from several populations after unrestricted feeding on oatmeal bait containing 0·005% warfarin for various numbers of days*

		Males					Females				
				Mean dose warfarin consumed (mg./kg.)					Mean dose warfarin consumed (mg./kg.)		
Animal source	No. of days feeding	Mean weight (g.)	Mortality	Survived	Died	Mean days to death	Mean weight (g).	Mortality	Survived	Died	Mean days to death
Merrick Landfill	1	372·3	4/12	1·9	2·2	6·8	236·6	1/11	2·4	1·5	8·0
	2	353·2	19/22	3·8	4·6	6·3	244·2	5/10	3·4	4·4	8·0
	3	261·6	10/10	—	7·4	5·5	282·9	10/10	—	7·0	8·3
Berlin disposal site	2	239·0	1/10	7·2	7·8	5·0	174·4	1/7	7·9	8·1	8·0
	3	372·4	9/10	6·7	6·5	6·8	273·5	7/8	3·3	6·1	7·3
	4	324·0	10/10	—	7·3	5·5	252·4	10/14	7·2	5·9	7·1
	5	290·2	5/5	—	12·7	5·6	248·3	9/9	—	10·3	6·7

Table 2. *Mortality to wild Norway rats from several populations after unrestricted feeding on oatmeal bait containing 0·005% warfarin for various numbers of days*

		Males					Females				
				Mean dose warfarin consumed (mg./kg.)					Mean dose warfarin consumed (mg./kg.)		
Animal source	No. of days feeding	Mean weight (g.)	Mortality	Survived	Died	Mean days to death	Mean weight (g.)	Mortality	Survived	Died	Mean days to death
Wyoming disposal site	6	308·3	10/10	—	7·4	6·4	216·9	7/9	11·6	10·2	7·0
	8	226·5	10/10	—	11·0	6·4	193·4	7/7	—	12·4	6·4
	10	210·4	8/8	—	12·0	6·3	204·9	8/8	—	11·8	6·8
Pittstown, disposal site (moderate poisoning pressure in recent past)	2	256·1	3/10	5·1	4·8	10·3	160·4	3/12	5·8	4·6	8·7
	3	255·1	9/15	6·6	7·4	7·2	161·2	5/12	7·5	9·0	8·8
	4	309·3	9/10	7·1	9·0	6·8	205·3	9/11	14·0	10·3	7·8
	5	328·1	11/11	—	8·6	6·3	194·9	9/10	13·6	12·4	7·2
	6	314·7	9/9	—	7·2	5·4	182·5	10/11	18·5	14·3	7·5
	7	—	—	—	—	—	228·6	14/14	—	14·3	6·7
Selkirk, chicken farm	6	280·2	12/12	—	10·8	6·1	186·1	24/27	12·1	14·1	7·5
Cambridge											
Farm A	6	179·0	2/3	16·4	13·5	9·0	188·8	5/5	—	14·4	7·8
Farm B	6	200·2	4/4	—	14·8	6·5	196·6	6/7	8·9	14·6	7·2
Turkey farm (long-term poisoning)	4	245·2	1/8	9·7	9·3	5·0	179·0	1/8	8·0	6·9	7·0
	6	274·9	6/8	9·2	8·4	6·5	211·7	5/8	9·2	11·9	7·2
	8	296·6	8/8	—	10·8	6·5	186·2	6/8	15·5	14·1	7·5
	10	307·3	12/12	—	10·6	7·3	186·7	10/12	23·5	16·1	7·8
	12	—	—	—	—	—	193·6	9/10	22·1	13·1	8·1

a 6-day feeding on 0·005% warfarin should give a 98% mortality with 95% confidence limits for both sexes. Both populations, then, confirm the usefulness of a 6-day feeding period as a screen for detecting resistant-suspect animals.

There is a clear difference in response of the sexes to warfarin. Females from both populations took significantly longer to die than did males. The mean day of death at Merrick to obtain a 100% mortality for males and females was $5{\cdot}5 \pm 0{\cdot}4$ and $8{\cdot}3 \pm 0{\cdot}6$ ($P = 0{\cdot}01$). At Berlin the respective means were $5{\cdot}5 \pm 0{\cdot}4$ and $6{\cdot}7 \pm 0{\cdot}4$ days ($P = < 0{\cdot}005$).

Resistant and resistant-suspect animals

Bentley (1969) has defined resistant rats in the United Kingdom as those that survived a standard feeding period of 6 days on 0·005% warfarin in the laboratory. Rats that meet this criterion have been found at two areas in eastern New York (Table 2). The first discovered of these populations was found on a turkey farm near Cambridge, New York. Some 80 rats were trapped from this farm over a 1-month period and returned to the laboratory for testing. The turkey farm from which the rats were collected had been poisoned with anticoagulants annually for the past 20 years. The general rural area was characterized by mixed agricultural operations, mainly animal production, such as turkeys, chickens, swine, beef cattle and dairies. Food crops, primarily corn, are grown on some acreages. Rats occurred on several nearby farms; on one of these (farm A) a male rat survived the largest dose thus far taken by a male. This farm, on which swine are raised, lies immediately adjacent to the turkey farm. The other farm (B) is 2 km. to the north-west and is a dairy. The one surviving female here was not considered exceptional.

The second site was a small chicken farm near Selkirk, New York. Some 40 animals were captured here during a month of trapping. This farm had a history of irregular poisonings with anticoagulants whenever the rat population reached intolerable levels. The dosages survived here indicate a very early development in resistance.

The feeding pattern described by Lund (1969), where the amount of food consumed by resistant rats on days 5 and 6 of the test was no less than 75% of that consumed on days 1 and 2, was not seen in any of the rats from the Cambridge or Selkirk areas. Instead, especially on the longer feeding periods, surviving rats interspersed feeding periods of 3–4 days with fasting periods of 2–3 days. This feeding pattern was observed in Norway rats by Drummond & Wilson (1968), who considered it perhaps of practical importance in the poisoning of wild populations.

Rats survived 6-day feeding tests from two other sites but these are considered as resistant-suspect populations only. At Pittstown, females survived doses at 4-, 5- and 6-day feeding periods that suggest emerging resistance. This site had been poisoned moderately with anticoagulants for a period of several years before the animals were tested. At Wyoming village two females survived the 6-day test but all died at 8- and 10-day offerings, so the population is regarded as unexceptional.

Table 3. *Daily intake of warfarin by wild Norway rats when allowed unrestricted feeding upon ground oatmeal containing* 0·005 % *warfarin*

Days of feeding	No. of animals	Mean intake of warfarin (mg./kg.)	Mean daily intake warfarin (mg./kg.)
1	23	2·2	2·2
2	71	5·4	2·7
3	65	7·1	2·4
4	61	8·4	2·1
5	35	10·7	2·1
6	113	11·7	1·9
7	14	14·3	2·0
8	33	12·8	1·6
10	40	13·1	1·3
Totals and means	455	9·3	1·9

DISCUSSION

Drummond & Wilson (1968) proposed the use of warfarin at 0·005 % in baits for susceptibility testing, using a 6-day feeding period as a screening test. Since that time only two papers have appeared using the proposed technique and, consequently, there is very little comparative data. Krishnamurthy, Uniyal & Pingale (1968) carried out susceptibility studies of *Rattus rattus* in Hapur, India, using 0·025 % warfarin in semolina. The roof rat, being considerably more tolerant of warfarin than *Rattus norvegicus*, required a 13-day feeding period for 100 % mortality. These results compare favourably with those obtained by Bentley & Larthe (1959) using the same warfarin concentration against *R. rattus*.

Telle (1971, 1972) reported the results of susceptibility studies in Norway rats in northern Germany. His results are remarkable in that the consumption of warfarin in mg./kg. of rat is quite low. Our observations indicate that an animal feeding upon ground oatmeal bait containing 0·005 % warfarin has a daily intake of anticoagulant ranging from 1 to 4 mg./kg. We found a mean daily intake of warfarin in 455 tested rats to be 1·9 mg./kg. (Table 3). During the course of a 6-day test, total dosage intake by individual animals ranged from 5·0 to 18·5 mg./kg. Drummond & Wilson (1968) present data indicating that their rats have an even larger warfarin consumption daily. Resistant rats are ascribed to populations on the Island of Norderney by Telle. This conclusion is not justified in the light of comparative data from the United Kingdom or the United States.

Our observations upon the occurrence of warfarin-resistant rat populations in New York have led us to believe that resistance may arise in any sizeable persistent rat population that is repeatedly exposed to anticoagulant treatments over a period of years or several decades. Animal operations, at least in this part of the north-eastern United States, seem to offer the best potential for the resistance phenomenon to develop. Additional sites and populations fitting the above description are being sought and studied.

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Comparison of the virulence for mice of *Salmonella typhimurium* given by the intraperitoneal and subcutaneous routes

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(*Received* 18 *July* 1972)

SUMMARY

Salmonella typhimurium strain 1566 administered to mice by the intraperitoneal route was considerably more lethal than the same dose administered subcutaneously. This could be measured in terms of mortality, infectivity and mean survival time of those mice that died.

INTRODUCTION

It is the general belief that localization of virulent bacteria in the tissues is beneficial to the host, whereas active dissemination and multiplication of pathogenic organisms within the body can be fatal. However, when certain pathogenic organisms are rapidly disseminated in the tissues of mice and guinea-pigs – for example, after an intravenous (IV) challenge – the mortality rate is considerably lower than when the challenge is administered by the intraperitoneal (IP) or the subcutaneous (SC) routes (Sobernheim & Murata, 1924; Lange & Gutdeutsch, 1928; Ørskov, 1940; Dutton, 1955). In the case of the organism *Salmonella typhimurium*, Dutton (1955) reported that the lethality of the organism for mice when challenged by the IV route was less than when the animals were challenged by the IP or SC routes. He found that although the mortality rates were the same after IP or SC challenge, the mean survival time of those mice that died after an IP challenge was longer than that for the group challenged by the subcutaneous route. This suggests that *S. typhimurium* given subcutaneously is more virulent for mice than when the same challenge dose is given intraperitoneally.

During the course of a study on *S. typhimurium* strain 1566 infection in mice, the observations of Dutton were not confirmed. The results are accordingly presented for consideration.

MATERIALS AND METHODS

Animals

Male Swiss white mice each weighing 19–22 g. were used.

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Challenge organism

S. typhimurium strain 1566 grown overnight in nutrient broth was washed and resuspended in sterile 0·1 M phosphate buffer at pH 8, and a dose of 20 organisms was injected into each mouse in a volume of 0·1 ml. The actual dose administered was verified by a surface viable count (Miles & Misra, 1938).

Two groups of 20 mice each were challenged by the IP route and the SC route respectively. In a third group of 20 mice the challenge inoculum was divided and one half was given IP and the other half SC to each mouse. The mice were observed daily for 28 days; deaths were recorded and the spleen and liver of each dead mouse was cultured to establish that the animal had died with an *S. typhimurium* infection. All survivors were killed on the 28th day after challenge and their spleens and livers were cultured to test for the presence of *S. typhimurium*.

Statistical analysis

The observed mortality in each group of mice was analysed by the chi-square test of probability using a formula that makes allowance for small numbers. The mean times to death of those mice that died in each group were analysed by the Students *t* test. When the values for *P* were 0·05 or less the results were considered to be statistically significant.

RESULTS

The detailed results of this experiment are shown in Table 1. With an IP challenge of 20 *S. typhimurium* the mortality was 65% and the mean time to death of those mice that died was 14 days. When the same dose was given SC the mortality was 15% and the mean survival time of those mice that died was 22 days. The difference between the results for the IP and SC experiments were statistically significant. Mice that received half the challenge dose by the IP route and the other half by the SC route showed a significantly reduced mortality of 25% when compared with the mortality of the group challenged intraperitoneally. The mean time to death was prolonged to 20 days but this result was not statistically significant. The IP challenge was more lethal than SC challenge, and when the challenge dose was divided equally and administered by both routes the animals showed a mortality rate and mean survival time that was intermediate between the corresponding values observed after IP and SC challenge. The infectivity rates also reflected this trend in that SC challenge resulted in a lower infectivity rate (70%) than the IP challenge (95%), while those mice receiving the challenge dose by both routes showed an infectivity rate (80%) that was intermediate between the other two. The reductions in infectivity were not statistically significant.

DISCUSSION

Earlier work has shown that some organisms, including *Salmonella typhimurium*, are more lethal for mice when administered by the subcutaneous route than by the intraperitoneal route (Dutton, 1955). However, the results reported above

Table 1. *Groups of* 20 *mice were challenged by different routes with* 20 Salmonella typhimurium *strain* 1566

(The experiment was terminated on the 28th day after challenge.)

Route of challenge	No. of deaths	Mortality (%)	Mean time to death (days)	No. of survivors infected	Infectivity (%)
Intraperitoneal	13/20	65	14	6/7	95
Intraperitoneal and subcutaneous	5/20	25	20	11/15	80
Subcutaneous	3/20	15	22	11/17	70

indicate quite clearly that an IP challenge with 20 *S. typhimurium* organisms of strain 1566 is more lethal for mice than a SC challenge with the same dose. It is interesting that when half the challenge inoculum was administered IP and the other half SC to the same mice, the virulence as measured in terms of mortality, infectivity and mean survival time of those mice that died was intermediate between the corresponding values for IP challenge on the one hand and SC challenge on the other.

It appears that rapid clearance and dissemination of a virulent dose of *S. typhimurium* from the intraperitoneal space augments the lethality for the mouse when compared with the outcome of a subcutaneous challenge with its slow rate of clearance. It is possible that the slower rate of release from a subcutaneous site permits the defence mechanism of the animal to deal effectively with the pathogen before the disease reaches an acute stage. Another possible factor is that the antigen is processed in different ways, depending on whether the challenge is administered by the SC or IP routes. In other words, after phagocytosis of challenge organisms injected subcutaneously, antigenic material released may be so modified as to be more efficient in the rapid stimulation of host defence mechanisms.

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Aminoadamantane-resistant strains of influenza A2 virus

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SUMMARY

After one passage of influenza A2/Singapore/1/57 virus in mice treated with 150 mg./kg./day of aminoadamantane, a partially drug-resistant strain of virus was detected in 1 of 12 mice. The isolation rate of aminoadamantane-resistant viruses increased to 8 after three passages in drug-treated mice. Some virus strains showed a 500-fold increase in resistance to aminoadamantane and to the structurally related compounds α-methyl-1-adamantane methylamine and 2-adamantanamine sulphate. No aminoadamantane-resistant viruses were detected after passage of influenza four times in mice treated with lower (15 or 1·5 mg./kg./day) concentrations of aminoadamantane. Aminoadamantane had no detectable effect on the development of lung lesions in mice infected with the drug-resistant influenza strain, whereas lung lesions were reduced in aminoadamantane treated mice infected with a control strain of influenza A2/Singapore virus. No differences were detected in the buoyant density in caesium chloride, morphology or serology between control and aminoadamantane-resistant strains of virus. These drug-resistant influenza viruses may be useful for detailed studies of the mode of action of aminoadamantane.

INTRODUCTION

1-Aminoadamantane hydrochloride has been shown to have a prophylactic and therapeutic effect (Galbraith, Oxford, Schild & Watson, 1970; Galbraith *et al.* 1971; Iezzoni, 1970) against influenza A2 infections in the general community. Acquired resistance to chemotherapeutic agents is an important problem in bacterial infections and studies have indicated that viruses can also acquire resistance to and dependence on antiviral compounds (Eggers & Tamm, 1961; Eggers & Tamm, 1963; Melnick, Crowther & Barrera-Oro, 1961; Renis & Buthala, 1965; Subak-Sharpe, Timbury & Williams, 1969). We have described previously the isolation of a strain of influenza A2 virus from infected mice being treated with aminoadamantane which showed a considerably increased resistance to the compound (Oxford, Logan & Potter, 1970).

Results are presented here showing the frequency of selection of such amino-adamantane-resistant strains and some of the biological characteristics of these

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drug-resistant influenza viruses. As well as the practical importance of the study of aminoadamantane-resistant influenza strains, such viruses would be useful for more detailed studies of the mode of action of aminoadamantane and this property of drug resistance may be useful as a marker for genetic studies with influenza viruses.

MATERIALS AND METHODS

Viruses

A mouse adapted strain of A2/Singapore/1/57 (H2N2) kindly supplied by Dr C. R. McDonald, Pfizer Ltd., Sandwich, Kent, was used. It had received four passages in eggs and 50 passages in mice. In our laboratory the virus received two further egg passages at limiting dilutions and was stored as an allantoic fluid pool at −80°C. For some experiments influenza virus was purified as described previously (Laver, 1969).

Chemicals

1-Aminoadamantane hydrochloride was kindly supplied by Dr A. Galbraith, Geigy (UK) Ltd., Macclesfield, Cheshire.

Quantitative haemadsorption (Q.H.) *test*

This test was used to measure the degree of inhibition of influenza viruses growing in tissue culture cells by aminoadamantane. The method described by Finter (1964) was followed with some modifications (Oxford, Potter & Logan, 1970). Aminoadamantane was diluted in twofold steps from 0·02 to 25 μg/ml. in mixture 199 and 1·5 ml. added per cell culture tube. After 15 min. incubation approximately 10 EID 50 of influenza virus per cell in 0·2 ml. was added to each tube. The cells were incubated for 19 hr. at 35–36°C. and then treated with a 0·5% suspension of guinea-pig red blood cells for 15 min. at 4°C. In control tissue culture tubes which were inoculated with virus only, the majority of the cells were infected and red blood cells haemadsorbed strongly to them. After two washings with phosphate-buffered saline pH 7·2 (PBS) at 4°C. to remove unadsorbed red blood cells, 1·5 ml. of deionized water at 37°C. was added per tube to lyse the haemadsorbed red blood cells. Eight tissue culture tubes of BSC–1 cells were used for each dilution of aminoadamantane and the relative haemoglobin concentrations were determined from O.D. readings at 410 nm. in a Unicam spectrophotometer. The optical densities were plotted against the concentration of aminoadamantane on a logarithmic scale, and the concentration of aminoadamantane inhibiting haemadsorption by 50% (inhibitory concentration: IC 50 in μg./ml.) was read off the resulting graph.

Production of drug-resistant strains by passage of influenza virus in aminoadamantane treated mice

The experimental protocol is summarized in Fig. 1. Adult Swiss white mice were inoculated intranasally under ether anaesthesia with 0·1 ml. of PBS, pH 7·2, containing 10^4 EID 50 of virus. Mice were killed after 72 hr., the lungs removed

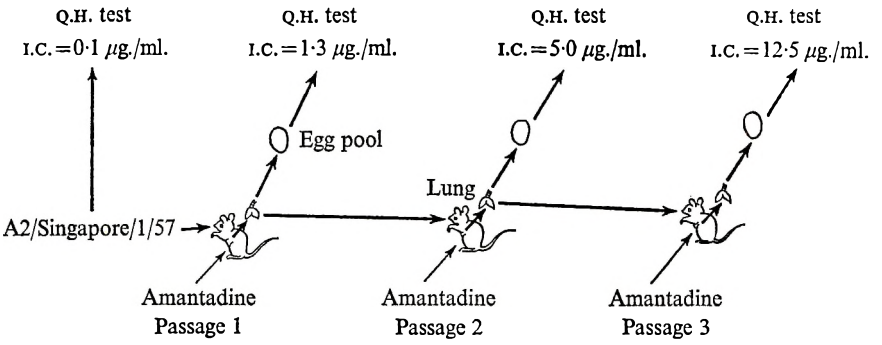

Fig. 1. Production of aminoadamantane- (Amantadine)-resistant strains of influenza virus.

aseptically and minced as a 10% (v/v) suspension in a mechanical blender in mixture 199. The suspensions were centrifuged at 2000 *g* for 10 min. and supernatant fluids frozen at −80°C. At each virus passage, mice were inoculated intraperitoneally with 50 mg./kg. of aminoadamantane 30 min. before inoculation with 10^4 EID50 of virus and supplied with drinking water containing 1 mg./ml. aminoadamantane. Separate tests established that mice received an approximate total of 150 mg./kg. aminoadamantane per day with this treatment.

Buoyant-density determination

One ml. of influenza haemagglutinin (diluted in 0·1 M tris HCl buffer, pH 7·4, to an HA titre of 1/128 approximately) was layered on the top of a preformed linear density gradient prepared from 2·4 ml. of 37% (w/v) and 2·1 ml. of 53% (w/v) CsCl dissolved in 0·1 M tris HCl buffer, pH 7·4. The tubes were centrifuged for 22 hr. at 100,000 *g* in the SW 39 rotor of a Spinco L preparative ultracentrifuge (Oxford & Potter, 1969).

RESULTS

The degree of inhibition of influenza viruses by aminoadamantane was estimated by quantitative haemadsorption (Q.H.). To determine the sensitivity and reproducibility of the Q.H. test two allantoic fluid pools of A2/Singapore/1/57 virus were tested for inhibition by the compound. A total of 13 Q.H. tests on different occasions using different batches of tissue culture cells with pool 1 gave a mean IC50 of 0·2 μg./ml. of aminoadamantane with a standard deviation of 0·19. Six Q.H. tests on influenza pool 2 gave a mean IC50 of 0·29 with a standard deviation of 0·10. The Q.H. test was thus a sensitive indicator of inhibition of virus growth and gave reproducible results.

Frequency of selection of aminoadamantane-resistant viruses

Influenza A2/Singapore/1/57 virus from a single egg pool was divided into 24 aliquots and these were used to infect 24 mice in individual cages. Twelve of these mice were treated with aminoadamantane (150 mg./kg./day) and the remaining 12 were control animals which received no drug. All mice were inoculated with

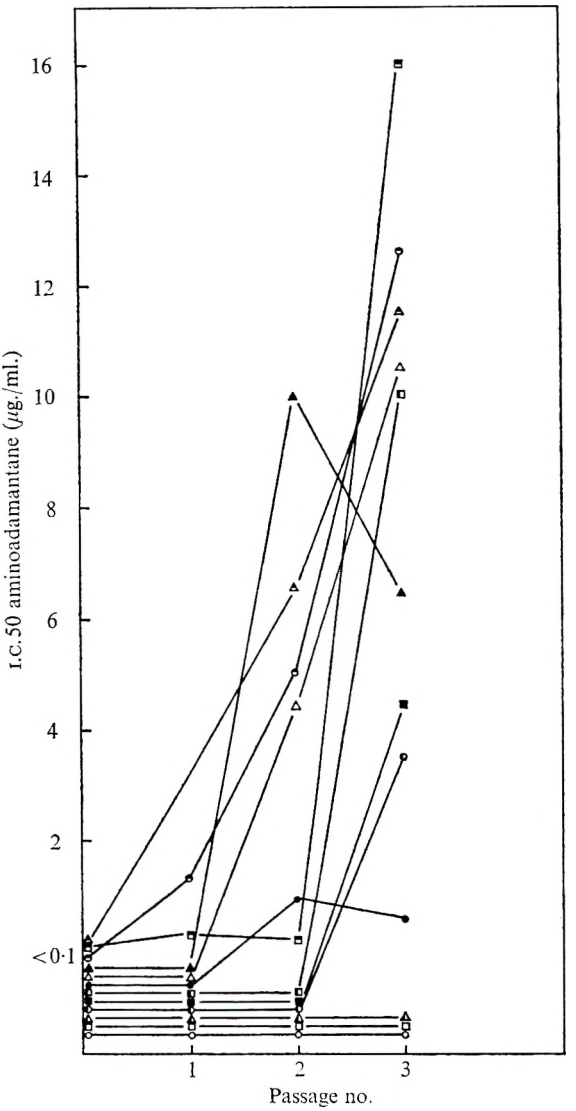

Fig. 2. Development of drug resistance during passage of influenza A2/Singapore/1/57 virus in aminoadmantane-treated mice.

approximately 10^4 EID 50 of virus intranasally. Lungs were harvested individually after 72 hr. and, after titration in eggs to determine the amount of infective virus, lung material was used to infect mice for the second passage (Fig. 1). After each of the three passages in mice, egg allantoic fluid pools were prepared from the infective lung material and the degree of inhibition of the isolated virus by aminoadamantane estimated by the Q.H. test.

The 36 virus pools from the control mice passage series were all inhibited by 0·02–0·2 μg./ml. of aminoadamantane in the Q.H. test. In contrast, influenza viruses resistant to inhibition by aminoadamantane were detected in a proportion of the 36 virus pools from drug-treated mice (Fig. 2). Drug-resistant viruses were

Table 1. *Production of aminoadamantane-resistant viruses in mice treated with different concentrations of aminoadamantane*

Dosage of aminoadamantane (mg./kg./day)	Proportion of drug-resistant viruses isolated after 4 passes in mice
150	6/9
15	0/9
1·5	0/7

detected in the lungs of 1 of 12 mice on the first passage of virus in aminoadamantane treated animals. After three passages in drug-treated mice the proportion of aminoadamantane-resistant viruses increased and resistant viruses were present in eight of the twelve passage series. A single virus strain showed partial resistance to inhibition by aminoadamantane after one passage in mice: before passage in mice growth of the virus in tissue culture was inhibited by 0·1 μg./ml. of aminoadamantane and after one pass in drug-treated mice 1·3 μg./ml. aminoadamantane was required for inhibition (Fig. 1). After two and three passes in drug-treated mice 5 μg./ml. and 12·5 μg./ml. of aminoadamantane respectively were required for inhibition. Three virus strains required two passages in drug-treated mice before showing any resistance to inhibition by aminoadamantane, while in four strains drug resistance only became apparent after three passages in treated mice. Thus, strains of A2/Singapore/1/57 virus resistant to inhibition by aminoadamantane could be obtained with relatively high frequency after several passages of virus in mice treated with high concentrations of aminoadamantane.

In a second series of experiments influenza A2/Singapore/1/57 virus was passaged four times in groups of mice treated with varying concentrations of aminoadamantane (Table 1). Drug-resistant viruses were recovered only from mice treated with large doses (150 mg./kg./day) of aminoadamantane; no drug-resistant viruses were recovered from mice treated with 15 or 1·5 mg./kg./day of aminoadamantane.

Biological and physical characteristics of an aminoadamantane resistant virus strain

Virus was examined from a single pool which had been passaged three times in drug-treated mice and required 12·5 μg./ml. of aminoadamantane for inhibition in tissue culture compared to 0·02 μg./ml. of aminoadamantane before passage in mice. This resistant strain was also resistant to α-methyl-1-adamantane methylamine and 2-adamantanamine sulphate, antiviral compounds structurally related to 1-aminoadamantane. Resistance to aminoadamantane was a stable property of the virus since the strain remained drug-resistant after passage four times in eggs at limiting dilutions in the absence of aminoadamantane. The virus pool used for the following experiments was cloned by two passages at limiting dilution in eggs.

The buoyant density of the aminoadamantane-resistant and the control strain of influenza A2/Singapore/1/57 was determined in linear gradients of caesium chloride (Fig. 3). No significant differences were detected between the two viruses and the mean buoyant densities from three experiments were 1·24 g./cm.^{3} and

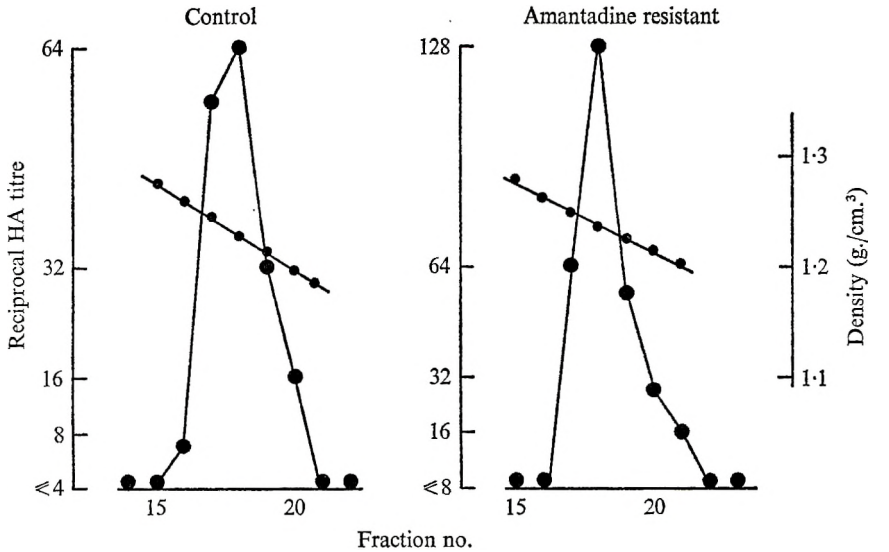

Fig. 3. Buoyant density in CsCl of aminoadamantane-resistant and control A2/Singapore/1/57 viruses. (A) Control virus. (B) Aminoadamantane-resistant virus.

1·23 g./cm.^{3} for the drug-resistant and the control strain respectively. Approximately 90% of virus haemagglutinin was recovered from the gradient with the control and aminoadamantane resistant strains. Electron microscopy (Oxford, Potter, McLaren & Hardy, 1971) showed preparations of the two strains purified by velocity-gradient centrifugation in sucrose to have a similar size and a non filamentous morphology typical of laboratory adapted strains of influenza virus.

No serological difference could be detected between the control strain and the drug-resistant strain. Haemagglutination and neuraminidase activities (Schild & Newman, 1969) of both viruses were inhibited to the same titre by a standard ferret antiserum* to influenza A2/Singapore/1/57 virus.

Finally, the aminoadamantane-resistant and the control virus were compared in *in vivo* studies to determine whether drug resistance was exhibited in animal experiments in addition to tissue culture (Table 2). The control strain of influenza A2/Singapore/1/57 caused lung lesions in mice 72 hr. after intranasal inoculation under ether anaesthesia and the amount of lung consolidation and the number of mice with lung lesions were both reduced significantly in mice treated with aminoadamantane. In contrast, aminoadamantane had no detectable effect on the incidence of lung lesions or on the degree of lung consolidation in mice infected with the aminoadamantane resistant strain of virus. Thus drug resistance was expressed in virus susceptible animals as well as in tissue culture.

Inhibition of influenza A2/Hong Kong/1/68 isolates by aminoadamantane

In an attempt to evaluate whether a proportion of naturally occurring influenza strains were resistant to inhibition by aminoadamantane, 30 freshly isolated

* Kindly supplied by Dr G. C. Schild, National Institute for Medical Research, Mill Hill, London, N.W.7.

Table 2. *Effect of aminoadamantane in mice infected with drug-resistant and control influenza* A2/*Singapore*/1/57 *viruses*

Virus	Treatment of mice	Lung lesion score (%) after infection with different virus dilutions: −1	−2	−3
Aminoadamantane-sensitive	0	52*	40	32
	150 mg./kg./day aminoadamantane†	27	16	0
Aminoadamantane-resistant	0	92	82	28
	150 mg./kg./day aminoadamantane	86	89	31

* Expressed as percentage of maximum score. Ten mice per virus dilution. Lungs examined 72 hr. after infection.

† Fifty mg./kg. of aminoadamantane 30 min. before infection and 1 mg./ml. aminoadamantane in drinking water.

Table 3. *Inhibition of influenza A2/Hong Kong/1/68 virus isolates by aminoadamantane*

Source of viruses	No. of viruses tested	IC50 conc. of aminoadamantane (μg./ml.) Range	Mean
Patients with influenza	26	0·02–0·26	0·09
Patients with influenza treated with 200 mg/day aminoadamantane	3	0·1–0·20	0·15
Contact with aminoadamantane-treated patient	1	0·08	–

strains of influenza A2/Hong Kong/1/68 virus were tested by quantitative haemadsorption (Table 3). All 30 strains were inhibited by 0·26 μg./ml. or less of aminoadamantane, including three strains which were recovered on the third day of illness from persons who had been treated with 200 mg. of aminoadamantane per day for 2 days, and one strain from a person in close familial contact with an aminoadamantane treated patient. Therefore no evidence of naturally occurring strains of influenza A2/Hong Kong/1/68 resistant to aminoadamantane was detected in this preliminary study.

DISCUSSION

The results indicate that drug-resistant strains of influenza A2/Singapore/1/57 virus can be selected with relatively high frequency after three passages in mice treated with high concentrations of aminoadamantane. The degree of drug resistance detected was considerable, particularly in viruses recovered after three passages in aminoadamantane-treated animals: some strains showed a 500-fold increase in resistance to the compound. However, no direct conclusions can be drawn from the present study about the possibility of emergence of aminoadamantane-resistant influenza strains in humans. The mice used in the present study were treated with relatively large concentrations of aminoadamantane (150 mg./kg./day) compared to the dosage in humans of 200 mg. per person per day. No drug-resistant strains were detected during passage of virus in mice treated with 15 or 1·5 mg./kg./day of aminoadamantane. Only three virus strains isolated from persons being treated with aminoadamantane were available for testing, and although these were all very sensitive to inhibition by aminoadamantane, further studies with many more strains are required.

Particularly interesting was the failure to detect differences in biological and physical properties between control influenza strains and aminoadamantane-resistant strains. Thus, the surface antigens of the two strains were similar. In addition, the buoyant densities in caesium chloride of the two virus strains were not significantly different. Aminoadamantane acts at an early stage of virus multiplication and may prevent virus penetration (Hoffmann, Neumayer, Haff & Goldsby, 1965) or uncoating (Kato & Eggers, 1969; Long & Olusanya, 1972) of the virus genome following the penetration step. Preliminary investigations have

failed to detect any effect of aminoadamantane on the activity of influenza-virion-associated RNA-dependent RNA polymerase (J. S. Oxford, in preparation). It might be expected that differences between the normal and drug-resistant viruses would be detected at the point of action of aminoadamantane; thus differences may occur in the method or rate of penetration or uncoating between the viruses. The use of aminoadamantane-resistant strains of influenza may help to elucidate the mode of action of the compound, and may lead to a more exact definition of the early stages of influenza infection in the cell.

The relatively mild antiviral activity of even large doses of aminoadamantane in mice and the break-through of influenza virus in treated tissue cultures (Oxford & Schild, 1968) noted in previous studies would seem to indicate a heterogeneous population of influenza virions containing a proportion of resistant particles. However, it is not possible to conclude from the results of the present study whether drug-resistant strains emerged by mutation and selection or more simply by selection from a heterogeneous virus population. Studies in tissue culture using plaque-purified viruses and more defined conditions of growth are required to answer these questions. Studies in humans have indicated that subclinical and clinical influenza may still occur in a proportion of persons receiving amino-adamantane prophylactically and that virus may be recovered from throat swabs in titres comparable to those from normally infected persons not receiving aminoadamantane (Iezzoni, 1970). Therefore it might be possible for any amino-adamantane-resistant virions selected in a person receiving the compound prophylactically or therapeutically to be subsequently transmitted. It is suggested that a surveillance of any possible emergence of drug-resistant influenza strains should be kept in future trials of aminoadamantane and other anti-influenza compounds.

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Mortality from cancer and cardiovascular diseases in the county boroughs of England and Wales classified according to the sources and hardness of their water supplies, 1958–1967

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SUMMARY

Relative rates of proportionate mortality from cancer of six sites based on total cancer deaths and the proportions expected in all towns, and from four types of cardiovascular disease based on total deaths from all causes, have been related in the 80 county boroughs of England and Wales to the sources of water supply and to the average hardness of water in the towns. The sources of water, from upland surfaces, artesian wells and rivers, were classified in eight groups, and significant associations were found for cancers of the stomach, oesophagus, prostate, male bladder and female breast, and for hypertensive and chronic rheumatic heart disease. No associations were apparent with intestinal cancer, vascular disease of the nervous system or arteriosclerotic heart disease. Hardness or softness of the water was classified in seven groups and significant associations were found for the same diseases as for source of water, none being evident for coronary disease.

INTRODUCTION

The concern of epidemiologists with water supplies to large towns began with studies of waterborne epidemics accidentally caused by pathogenic organisms, and interest was then taken in goitre and diseases due to a deficiency of some mineral element. Some attention has been directed to cancer of the stomach since a connexion with geology had been demonstrated, and recently an association has been suspected between softness of water and mortality from ischaemic heart disease. The present paper examines in greater detail than hitherto the mortality from cancer of several sites and from different forms of cardiovascular disease in 80 large towns in relation with their sources of water supply and water-hardness in the period 1958–1967.

ASSESSMENT OF MORTALITY BY PROPORTIONATE INDICES

The best method would be to calculate the mean annual standardized death rates for each sex, or the ratio of actual to expected deaths (SMR), but the populations and deaths by sex and age are not available from the Registrar General's annual reviews in respect of separate county boroughs but only the deaths by

cause at all ages for each sex, and the total populations without distinction of sex. Death rates at all ages combined can be fallacious when comparing towns since the age constitution of their populations can differ considerably, but alternative methods are possible for diseases which cause the great bulk of their mortality after age 45.

For comparing frequencies of *cancers* of different sites in groups of towns, for most of the sites the age distribution of deaths resembles that of total cancer so closely that a proportionate cancer rate (PCR) per 1000 deaths from all cancer in the same town provides a measure of relative mortality which is not appreciably affected by peculiarities in the distribution by ages. The average PCR of the towns forming a group can then be expressed as a percentage of the average PCR for all county boroughs, and this Proportionate Cancer Index (PCI) has been used for the various cancer sites in the tables which follow. These do not include lung, uterus or leukaemia, whose age distributions may differ sensibly from that of all cancer combined.

For the cardiovascular diseases a proportionate mortality rate per 1000 deaths from all causes (PMR) can be used when comparisons between groups of towns are needed and the data do not distinguish age at death. This is because few of the deaths from these causes occur before middle age and the PMR at all ages varies between groups of towns in almost the same way as would a PMR at all ages over 45. When expressed as a percentage index (PMI) of the average PMR in all county boroughs the resulting patterns of indices approximate closely to one another, showing that the disturbing effects of differing age distributions have been virtually eliminated. To demonstrate this, Table 1 shows the deaths from all causes and arteriosclerotic heart disease (International No. 420) for each sex at all ages and at all ages over 45 in the five conurbations which contain county boroughs in the year 1961.

The PMR's at all ages ranged from 177 to 236 per 1000 for males, and from 119 to 163 for females in the five conurbations. Expressing the PMR for males in terms of the average rate of 208·3 taken as 100, the resulting indices (PMI) for males at all ages ranged from 85 to 114, and expressing the PMR's for females in terms of the average 143·5, the resulting indices at all ages range from 83 to 113. When this is repeated with the PMR's at ages after 45 it is apparent from the final columns that in no case does the resulting PMI differ from the corresponding index at all ages by more than 2 %. The differences are unimportant and it follows that for the cardiovascular diseases whose mortality occurs chiefly after age 45 the proportionate mortality index for groups of large towns expressed in terms of the average for all county boroughs taken as 100 is virtually unaffected by disturbing effects of differing age distributions in the local populations and can be regarded as equivalent to the standardized mortality ratio (SMR). The PMI index at all ages, which is simple to calculate, can be used where comparisons between cardiovascular disease mortalities are being made from data where information as to ages at death or of the local population is not sufficient to allow assessment of standardized death rates or SMR's. The PMI index has been used for comparing the groups of county boroughs when classified according to their sources of water

Table 1. *Proportionate mortality rates and indices for arteriosclerotic heart disease at all ages and at ages after* 45 *in five conurbations in* 1961

Conurbations	Sex	Deaths from all causes at		Deaths from A.H.D. at		PMR per 1,000 at		PMR (% of average for 5 conurbations) at	
		All ages	Ages after 45	All ages	Ages after 45	All ages	Ages after 45	All ages	Ages after 45
Tyneside	M	5,305	4,717	1120	1078	211·1	228·5	101	102
	F	4,718	4,344	749	739	159·8	170·4	111	111
West Yorkshire	M	11,428	10,307	2701	2613	236·3	251·6	114	112
	F	11,417	10,773	1867	1860	162·6	172·6	113	114
South-East Lancashire	M	16,551	14,729	3275	3173	197·8	212·5	95	95
	F	16,201	15,088	1932	1907	119·3	126·4	83	82
Merseyside	M	8,523	7,522	1867	1810	219·1	239·3	105	107
	F	8,221	7,477	1262	1254	153·5	167·7	107	109
West Midland	M	13,266	11,787	2352	2262	177·3	192·6	85	86
	F	12,082	11,031	1469	1457	121·6	132·1	85	86
Average of 5 PMR's	M	—	—	—	—	208·3	224·9	—	—
	F	—	—	—	—	143·5	153·8	—	—

and hardness of water in Tables 6–7. To recapitulate, the index figure PMI for a group of towns (e.g. group 7 supplied by boreholes in underlying chalk deposits) is 100 × average of the PMR's of the 11 towns comprising the group/average of the PMR's for that sex in all the 80 county boroughs of England and Wales.

SOURCES AND HARDNESS OF MAIN WATER SUPPLY

Two factors which have been related to the mortality from ischaemic heart disease are softness of water and rainfall in the area using the supply, and it has been concluded from partial correlation in a number of large towns that the apparent effects of softness are secondary to those produced by rainfall (Roberts & Lloyd, 1972). It has to be remembered, however, that the hardness of tap-water depends on the origin of the water and on modifications produced in the course of the journey from source to tap, including modifications made deliberately by the water company. The effects of rainfall depend on the average amount of rain falling upon the catchment area, which may be very different from that on the towns using the water.

Neither of these factors takes account of the mineral and organic substances other than calcium which find their way into the water supply, some present in the strata from which an artesian supply is drawn, others washed from upland surfaces into the reservoirs and derived from peat, bracken, organic carbon and mineral derivatives of the topsoil and from atmospheric pollution and fall-out over the gathering-ground. Where the main supply is obtained from boreholes into deep strata most of this contamination is likely to be avoided, and this might account for the higher mortality from stomach cancer in towns supplied by upland surface water in Northern England compared with that in towns supplied by boreholes in chalk or sandstone in the South. This suggests that some specific substance affecting the incidence of stomach cancer and washed from the moorland slopes might be responsible for the contrast, but it will be seen from Table 2 that the picture is complicated by other factors affecting the North Midland area which preclude a definite conclusion on this question.

In the analysis which follows, the sources of water supply in the 80 county boroughs have been classified into groups, and the mortality indices in the groups for 1958–67 have been examined for each cause of death, whilst a separate and independent grouping according to hardness of the water was also made.

The classifications employed for the two factors are shown below, eight groups being used for source of water and seven for hardness. The latter grouping corresponds with the divisions used by the Water Boards, namely: S, soft (0–50 ppm); MS, moderately soft (50–100), SH, slightly hard (100–150); MH, moderately hard (150–200); H, hard (200–300); VH1, very hard (over 300 ppm) (with subgroup VH 2 for magnesian limestone origin).

Classification of county boroughs by source of water supply and hardness

1. *Boreholes in magnesian limestone*

VH 2
West Hartlepool
South Shields
Sunderland

2. *Boreholes in sandstone ('Bunter' deposits) in North Midlands and South Lancashire*

MS	MH	H	VH 1
St Helens	Walsall	Warrington	Wolverhampton
	Smethwick	Dudley	
	West Bromwich	Stoke-on-Trent	

3. *Upland surface sources from Pennine area*

S	MS	SH
Huddersfield	Rotherham	Newcastle-on-Tyne
Bradford	Middlesborough	Gateshead
Preston	Leeds	Tynemouth
Halifax	Blackpool	Burnley
Wakefield	Sheffield	
Oldham	Barnsley	
Dewsbury	Blackburn	
	Rochdale	

4. *Upland surfaces and reservoirs in Lake District*

S	MS
Manchester	Bolton
Salford	Bury
Barrow-in-Furness	

5. *Upland surfaces and reservoirs in Wales and Devon*

S	MS	SH
Merthyr Tydfil	Cardiff	Chester
Birmingham	Newport	Swansea
Liverpool	Coventry	
Birkenhead	Exeter	
Bootle	Plymouth	

6. *Boreholes in New Red Sandstone in southern regions*

S	SH	MH	H	VH 1
Stockport	Wallasey	Burton-on-Trent	Lincoln	Southport
	Nottingham	Derby	Bath	Gloucester
	Wigan		Bristol	
			Doncaster	

7. *Boreholes in chalk deposits*

MS	H	VH 1
Hastings	Eastbourne	Kingston-on-Hull
	Bournemouth	
	Southampton	
	Reading	
	Brighton	
	Canterbury	
	Grimsby	
	Ipswich	
	Portsmouth	

8. *Rivers*

S	MS	MH	H	VH 1
Oxford	Carlisle	Northampton	Worcester	Great Yarmouth
		Darlington	York	Norwich
		Southend-on-Sea		
		Leicester		

Notes on above grouping. In Tables 2 and 4–7, groups 1 and 2 are placed first on account of their peculiar features. Group 1 comprises the three Durham towns drawing their water from magnesian limestone deposits (also separated as VH 2 in the hardness table). Group 2 comprises the towns in Staffordshire and adjacent areas taking their water supply from boreholes in the underlying sandstone which forms part of the New Red deposits of Triassic age which have an offshoot into South Lancashire. The Staffordshire towns have abnormal features in their populations affecting mortality which are not connected with the water supply (as will be seen from Table 3). Groups 3, 4 and 5 derive their water from upland surfaces, and groups 6 and 7 from boreholes or wells in chalk or sandstone in the south of England. Towns supplied mainly by river water are assembled in group 8 and are mostly in the south except Carlisle, Darlington and York.

MORTALITY IN 1958–67 FROM TEN CAUSES OF DEATH IN COUNTY BOROUGHS OF ENGLAND AND WALES

(*International Numbers of the diseases in parentheses*)

The proportionate mortality averages (per 1000 total cancer deaths for the six sites of cancer and per 1000 deaths from all causes for the four cardiovascular diseases) are compared by source of water supply and hardness of water for each cause of death. In the tables which follow the average PCR (or PMR in Tables 6–7) is expressed as an index (PCI or PMI) in terms of the average rate (E) for all the 80 county boroughs taken as 100, so that the presence of any appreciable relationship with the kind of water source or with the water hardness is easily seen.

Table 2 shows that hard water coming from magnesian limestone deposits (VH 2) is associated with cancer indices of 114 and 126 for the stomach but that other hard waters were characterized by indices below the average for all county boroughs. Disregarding the first two anomalous groups for source of water supply, the distribution shows a strong relation with the index levels. For males the rates are high in towns deriving water from upland slopes in the Pennine and Lake District areas and low in the southern towns served from artesian wells in chalk and sandstone or from rivers whose water is of mixed surface and deep origin. For females the indices are 104–107 for the upland groups 3–5 compared with 79 and 82 in the chalk and sandstone districts of the south. This suggests that water draining from mountain slopes contains chemical substances which are not present to the same extent in the deep-water supplies obtained through artesian wells and boreholes.

The statistical significance of the association with source of water is beyond question, as shown by aggregating the squares of the differences (d) from the expected value of 100, dividing by 100 and multiplying by n, the number of towns in the group, and comparing the mean value of the total ($S(nd^2/100)/8$) with the value of P, the probability of such a distribution occurring by chance. The result is 12·9 for males and 34·2 for females, for which P is in each case less than 0·001. The distribution of mortality indices according to the hardness and softness of water in the seven groups gives mean values of $nd^2/100$ of 6·9 for males and 11·1 for

Table 2. *Cancer of stomach* (151). *Relative proportionate mortality* (P.C.I.)

		Average PCR (% of expected value (*E*) in towns grouped by source of water)							
		Boreholes in North		Upland surface waters			Boreholes in South		
Sex	Average PCR in all County Boroughs (*E*)	Dolomite deposits (1)	N. Midland Sandstone (2)	Pennine slopes (3)	Lake District (4)	Wales; Devon (5)	New Red Sandstone (6)	Chalk deposits (7)	River water (8)
M	151	114	118	116	105	98	91	88	88
F	138	126	133	106	107	104	82	79	85

		Average PCR (% of expected value (*E*) in towns grouped by hardness of water)						
							Very hard	
		Soft (S)	Moderately soft (MS)	Slightly hard (SH)	Moderately hard (MH)	Hard (H)	VH 1	VH 2
M	151	100	108	101	103	90	91	114
F	138	105	101	104	107	86	95	126

Table 3. *Cancer of stomach and breast in females. Proportionate mortality indices in North Midland and West Riding towns*

North Midland	Hardness	Cancer of Stomach (PCI)	Cancer of Breast (PCI)
Walsall	MH	157	134
Wolverhampton	VH 1	135	132
Smethwick	MH	114	122
West Bromwich	MH	168	138
Dudley	H	137	132
Stoke-on-Trent	H	119	99
Warwickshire			
Birmingham	S	98	108
Coventry	MS	101	108
West Riding			
Huddersfield	S	81	92
Bradford	S	88	98
Barnsley	MS	105	86
Dewsbury	S	112	83
Halifax	S	136	93
Wakefield	S	119	100

females, corresponding with $P < 0{\cdot}01$ and $P < 0{\cdot}0001$ respectively – both significant at the conventional level. The strength of the associations with mortality appears to be greater for females than for males.

Whatever the substances in the water may be which affect stomach cancer, they do not affect the other cancers identified in this study but appear to be specific for stomach. This can be seen, for example, for intestine and rectum by ranking the 80 towns in order of their PCR's for stomach in females and noting that the average indices in years 1963–7 for the intestine show no correspondence with the stomach rates. For the 20 towns with lowest rates (average stomach PCR 99 per 1000 total cancer) the average intestinal rate was 167 and for the 20 towns with highest stomach rates (PCR 166) the average intestinal rate was 173. There is no tendency therefore for intestinal cancer to be more frequent in towns where the stomach cancer rate is high than it is where the stomach rate is low. The local factors affecting the stomach evidently do not affect the intestine and rectum.

In Table 2 the indices for group 2, which consists of the six county boroughs of Staffordshire with the adjacent towns of St Helens, Warrington and Dudley, deriving their water supply from the New Red Sandstone underlying the area, are remarkably high compared with the other groups (PCI 118 for males and 133 for females). A similar anomaly is seen for breast cancer in Table 5 (index 115), hypertensive heart disease in females (117) and chronic rheumatic heart disease in males (118 in Table 7).

Roberts & Lloyd (1972) commented on the low rates of mortality from ischaemic heart disease in the Staffordshire towns with their hard water supplies contrasted with Birmingham with its soft water from Wales. To elucidate the curious figures

for stomach and breast cancers Table 3 shows the mortality indices for females in the Staffordshire towns compared with Birmingham, Coventry and six West Riding towns to the north which are also supplied by soft water. In the Staffordshire towns indices for stomach cancer range from 114 to 168 and for breast from 122 to 138 apart from Stoke-on-Trent with index 99. In the six West Riding towns the stomach indices ranged from 81 to 136. Birmingham and Coventry had normal rates for stomach and slight excess for breast. In the absence of evidence that anything in water supplies has a specific effect on breast cancer mortality it must be concluded that the high rates for both stomach and breast in the North Midland towns are due to factors not connected with water such as peculiarities in the population arising from their high proportions of immigrants. The level of the general death rates in the six towns is relevant in this connexion. Since 1951 their rate, adjusted for age and sex, has been 11% in excess of that in all county boroughs and in Birmingham, and more than 15% above the national rate, and it cannot be supposed that this had anything to do with the water supply.

Contrasted with stomach, cancer of the intestine and rectum in females shows no association with source of water ($P = 0{\cdot}17$) or hardness of water ($P = 0{\cdot}30$), except in the groups of towns deriving water from boreholes in magnesian limestone, which had a low index of 84 (groups 1 and VH 2), this being possibly due to a medicinal effect. Rates for this site of cancer are for 1963–7.

Cancer of the oesophagus shows high indices in towns of group 5, with sources of water from upland surfaces of Wales and Devonshire. Exeter and Plymouth had 144 and 112 respectively for males and 115 and 147 for females, as had Birkenhead (152, 126) and Chester (154, 108), whilst female rates in Cardiff and Swansea were also high (133, 145). In group 4, with Lake District water, Salford (113), Bury (126) and Barrow-in-Furness (151) gave indices over 100 for females, but the Manchester index was normal. The association with water source was significant ($P < 0{\cdot}01$) for males, as well as for females ($P < 0{\cdot}005$). There was also a significant association with water hardness for females ($P < 0{\cdot}001$).

Cancer of the breast in Table 5 shows significant variation from expectation for females. The mean value of the summation of $100n(d^2/E)$ was 9·3, corresponding to $P < 0{\cdot}005$ for source of water supply, and 6·7 ($P < 0{\cdot}01$) for hardness of water. The groups with high indices for water source were nos. 2 and 6, where the water is obtained by boreholes in the New Red Sandstone in the North Midland area and the South, with indices 115 and 118. The towns with moderately hard and very hard water showed indices of 120 and 109. This curious relation with the sandstone has been examined in Table 3.

Cancer of the prostate in Table 5 showed a significant variation from expectation for source of water ($P < 0{\cdot}001$). High proportionate mortality occurred in group 7, where water is obtained mainly by boreholes in chalk (PCI 122). Of the 11 towns of this group 8 had indices of 105 or more (Hastings 172, Eastbourne 155, Bournemouth 139, Portsmouth 136, Southampton 126, Reading 114, Grimsby 110, Brighton 109). Low mortality was seen in group 4, with Lake District water (Manchester 72, Salford 80, Barrow-in-Furness 90), and in group 1 the magnesian limestone towns with index 88 (South Shields, West Hartlepool 79, Sunderland 91).

Table 4. *Cancer of oesophagus* (150), *intestine and rectum* (152–4). *Relative proportionate mortality* (P.C.I.)

Sex	Average PCR in all County Boroughs (*E*)	Average PCR (% of expected value (*E*) in towns grouped by source of water)							
		Boreholes in North		Upland surface waters			Boreholes in South		
		Dolomite deposits (1)	N. Midland Sandstone (2)	Pennine slopes (3)	Lake District (4)	Wales; Devon (5)	New Red Sandstone (6)	Chalk deposits (7)	River water (8)
		Cancer of oesophagus							
M	26·0	99	87	92	92	108	105	103	101
F	23·8	98	91	92	115	116	94	95	95
		Cancer of intestine and rectum							
F	16·8	84	106	97	97	105	104	100	99

Sex	Average PCR in all County Boroughs (*E*)	Average PCR (% of expected value (*E*) in towns grouped by hardness of water)						
		Soft (S)	Moderately soft (MS)	Slightly hard (SH)	Moderately hard (MH)	Hard (H)	Very hard	
							VH 1	VH 2
		Cancer of oesophagus						
M	26·0	94	95	101	106	94	98	103
F	23·8	103	112	91	82	97	97	98
		Cancer of intestine and rectum						
F	16·8	97	100	102	98	104	98	84

Table 5. *Cancer of breast* (170), *bladder* (175), *and prostate* (177). *Relative proportionate mortality* (P.C.I.)

Sex	Average PCR in all County Boroughs (*E*)	Average PCR (% of expected value (*E*) in towns grouped by source of water)							
		Boreholes in North		Upland surface waters			Boreholes in South		
		Dolomite deposits (1)	N. Midland Sandstone (2)	Pennine slopes (3)	Lake District (4)	Wales; Devon (5)	New Red Sandstone (6)	Chalk deposits (7)	River water (8)
		Cancer of breast							
F	192	87	115	94	91	100	118	95	105
		Cancer of bladder							
M	41·3	97	78	107	73	95	96	94	106
F	19·5	99	92	98	103	108	98	105	98
		Cancer of prostate							
M	62·3	88	91	94	84	99	99	122	104

Sex		Average PCR (% of expected value (*E*) in towns grouped by hardness of water)						
		Soft (S)	Moderately soft (MS)	Slightly hard (SH)	Moderately hard (MH)	Hard (H)	Very hard	
							VH 1	VH 2
		Cancer of breast						
F	192	96	98	95	120	98	109	97
		Cancer of bladder						
M	41·3	101	92	106	97	116	100	97
F	19·5	107	98	107	94	99	88	99
		Cancer of prostate						
M	62·3	89	99	97	102	112	105	88

Table 6. *Vascular disease of central nervous system* (430–434). *Relative proportionate mortality*

		Average PMR (% of expected value (*E*) in towns grouped by source of water)							
		Boreholes in North		Upland surface waters			Boreholes in South		
Sex	Average PMR in all County Boroughs (*E*)	Dolomite deposits (1)	N. Midland Sandstone (2)	Pennine slopes (3)	Lake District (4)	Wales; Devon (5)	New Red Sandstone (6)	Chalk deposits (7)	River water (8)
M	102·4	90	96	105	93	93	100	103	106
F	174·0	97	99	100	96	94	102	104	108

		Average PMR (% of expected value (*E*) in towns grouped by hardness of water)						
							Very hard	
		Soft (S)	Moderately soft (MS)	Slightly hard (SH)	Moderately hard (MH)	Hard (H)	VH 1	VH 2
M	102·4	97	102	102	107	100	98	90
F	174·0	96	101	101	102	103	97	97

In other groups of towns indices below 120 occurred for Coventry (147), Exeter (144), Bath (146), Newcastle (137), Wakefield (124) and Nottingham (138). Water hardness did not appear to have any important association with prostatic cancer ($P < 0{\cdot}01$).

Cancer of the bladder in males showed very low mortality in group 2, the sandstone area in the North Midlands, and in group 4 with Lake District source, and hard water may have been concerned in this (index 116 for group H). Bladder cancer in women showed no appreciable association with water source but the index was 107 in groups S and SH. The associations were significant for males ($P < 0{\cdot}01$) but not for females.

It was pointed out in 1947 that male cancers of the bladder, prostate and rectum had low standardized death rates in 1921–30 in the county boroughs with a high average annual rainfall over 30 in., and high rates in towns with averages below 25 in. The reverse was true of skin cancer and it was suggested that greater dryness of the air resulting in more excretion of moisture from the skin and less from the other organs produced a greater liability in the latter (Stocks, 1947). Evidently, from Table 5, softness and hardness of drinking water is not a factor of importance.

In Table 6 there is no evidence of association between mortality from vascular lesions of the central nervous system (430–434) and source of water supply. The values of P according to the d^2/E test were above the conventional level of 0·05 for each sex. The only group indices over 105 were for the towns using river water (group 8). Other towns with rates above 110 were Darlington (males 123, females 121), Leicester (136, 124), Carlisle (118 for females), Northampton (116 for males), Worcester (117 for females). The lowest index was for the dolomite group 1 in males. Water hardness groups also showed no significant association with mortality.

The only indication in Table 7 of any connexion between arteriosclerotic disease mortality (420) and source of water supply is the low index for each sex in group 2 (males 88, females 87). The six towns in this group supplied by sandstone underlying the North Midland area had low proportionate indices, namely West Bromwich (93, 61), Wolverhampton (68, 80), Walsall (84, 83), Dudley (85, 97), Stoke-on-Trent (88, 100), Smethwick (107, 87), these PMI figures being for males and females. The low levels contrast with the high indices for cancer of the stomach and breast in females shown in Table 3. The test for differences from expectation in the whole distribution of groups gave $P = 0{\cdot}15$ for males and $P = 0{\cdot}05$ for females (not significant).

In an analysis of standardized mortality during 1954–8 in the National Atlas of Disease Mortality (Howe, 1963) it was observed that the town with the highest rate for coronary disease was Halifax, with SMR for males 160 and females 163 (based on national rates inclusive of the large towns). In the present study for 1958–67 this was also true, the PMI being 133 for males and 155 for females, whilst other towns giving male indices above 110 (in terms of all county boroughs taken as 100) were Wallasey, Great Yarmouth, Carlisle, Barrow-in-Furness, Huddersfield, Bournemouth, Southend, Blackpool, Southport, Cardiff, Swansea, Oxford, Coventry, Eastbourne and Leeds.

Table 7. *Arteriosclerotic heart* (420), *hypertensive heart* (440–443) *and chronic rheumatic heart disease* (410–416). *Relative proportionate mortality*

Sex	Average PMR in all County Boroughs (*E*)	Average PMR (% of expected value (*E*) in towns grouped by source of water)							
		Boreholes in North		Upland surface waters			Boreholes in South		
		Dolomite deposits (1)	N. Midland Sandstone (2)	Pennine slopes (3)	Lake District (4)	Wales; Devon (5)	New Red Sandstone (6)	Chalk deposits (7)	River water (8)
		Arteriosclerotic heart disease							
M	222	99	88	103	94	102	99	101	105
F	151	108	87	105	94	100	91	102	102
		Hypertensive heart							
M	23·7	97	82	75	78	116	94	111	93
F	32·6	111	117	90	97	117	87	100	102
		Chronic rheumatic heart							
M	8·2	92	118	108	102	99	99	88	88
F	17·2	102	105	112	117	117	95	72	95

		Average PMR (% of expected value (*E*) in towns grouped by hardness of water)						
		Soft (S)	Moderately soft (MS)	Slightly hard (SH)	Moderately hard (MH)	Hard (H)	Very hard	
							VH 1	VH 2
		Arteriosclerotic heart disease						
M	222	102	102	100	95	100	96	99
F	151	102	102	100	92	101	98	108
		Hypertensive heart						
M	23·7	87	90	117	111	112	84	97
F	32·6	95	97	105	102	107	88	111
		Chronic rheumatic heart						
M	8·2	101	107	100	112	90	80	92
F	17·2	111	112	112	94	78	90	100

There is no indication of any association with hard or soft water except possibly the female index of 108 for the magnesian limestone area of group 1. If the rainfall map of England and Wales (Stocks, 1937) is compared with the geographical distribution of coronary disease a tendency is seen for the county boroughs in areas with high annual rainfall to have higher rates for coronary disease, but the softness of the water supply as distributed in Table 7 shows no obvious connexion with this. The distribution according to hardness group shows no significant departure from expectation, the probabilities of the observed variation occurring by chance being $P = 0{\cdot}45$ for males and $P = 0{\cdot}25$ for females. It must be concluded from this study of the 80 county boroughs of England and Wales that no appreciable connexion exists between their mortality from coronary disease during 1958–67 and the hardness or softness of their water supplies. The recent conclusion in a paper by Roberts & Lloyd (1972) after applying partial correlation to data from towns in South Wales and England, that the apparent association between death rates from ischaemic heart disease and softness of water was secondary to the rainfall level, is compatible with what is shown in Table 7. The rather prevalent belief that soft water is a major factor in the incidence of coronary disease cannot be sustained without more convincing evidence than exists at present.

The most curious features of the distributions in Table 7 for hypertensive heart and hypertension without mention of heart (440–443), which differs from expectation for source of water ($P < 0{\cdot}001$ for each sex), are (1) the high indices for the towns provided by upland surface water from Wales for both sexes in group 5 with PMI levels 116 and 117, (2) the very low rates for males in towns supplied by water from the Pennine slopes (group 3, with index 75) and Lake District (group 4, index 78), and (3) the contrasted male and female indices (82, 117) for the North Midland group supplied by boreholes in sandstone. This could account for the contrast between the high index for males in Birmingham (120) and low index in Manchester (70), but complex factors are involved in the diagnosis and certification of this cause of death which may be important. Other towns with indices above 110 for both sexes, taking their water supply from Wales, were Cardiff (151, 159), Swansea (151, 159) and Merthyr Tydfil (148, 158), and for females in Bootle (141) and Chester (134), but the indices for Liverpool and Birkenhead were below 100. The index for the chalk group 7 was 111 for males but low for females, those with indices over 110 being Canterbury (187), Grimsby (154) and Southampton (127).

The table for water hardness shows high rates where the water was hard (SH, MH, H), particularly for males, and the dolomite group gave a high index for females but not for males. The test for significance of the differences from expectation gave $P < 0{\cdot}001$ for males and $P = 0{\cdot}04$ for females. There is evidently a tendency for hypertensive heart mortality to be high where the water is hard despite the absence of any such tendency for coronary disease.

Chronic rheumatic heart disease as cause of death (410–416)

In Table 7 there is a peculiarly high frequency for males in group 2, the towns in the North Midland area supplied by artesian wells and boreholes in the sandstone

underlying that area, with index 118, and the industrial towns supplied by upland surface water from the Pennine slopes in group 3 also show a rate above expectation (108). The distribution is quite different from those for coronary disease and hypertensive heart. In females the towns with high indices were those supplied by upland surface water in groups 3, 4 and 5, with indices 112, 117 and 117, the excess in the North Midland towns being only slight (105). In both sexes the chalk and river groups had low levels of mortality, contrasting with the other heart groups. The distributions for both sexes differ significantly from expectation, with probability values $P < 0{\cdot}01$ and $P < 0{\cdot}001$ for males and females.

Hardness of water showed high frequency in the soft-water groups for females and low indices for the hard and very hard groups, but in males the association was less evident. As for source of water, the differences from expectation are significant, with $P < 0{\cdot}001$.

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Age, time and cohort factors in mortality from cancer of the cervix

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SUMMARY

Data for mortality from cancer of the cervix in England and Wales by 5-year age groups and four quinquennia (1951–70) are analysed. The logarithms of the mortality rates are regressed on age group, epoch of death and epoch of birth. The factors obtained are considered in relation to particular features of the mortality pattern, such as the reversal of trend in certain age groups.

INTRODUCTION

Patterns of mortality from cancer of the cervix present a number of peculiar features. Primarily a disease of the human species, its frequency in relation to all cancer in females ranges from 5 to 60% in different population groups, and when one reflects on the lower frequencies found, *inter alia*, in developed countries, rural populations, high socio-economic levels, Jewish women, the Amish women, and single women, as compared to higher frequencies in developing countries, urban populations, low socio-economic levels, negresses, married women and other associations (Dunham, 1968; Voors, 1967), one is led to suspect a local cause in women who later develop the disease.

Hill & Adelstein (1967), presenting data for mortality from cervical carcinoma, 1951–65, England and Wales, showed that, although a downward trend existed in the standardized mortality rates, rises in age-specific death-rates had occurred in certain age groups, beginning with those aged 30–34 and later changing to older groups up to ages 50–54. Similar intrusions in the generally falling rates were reported by Adelstein, Hill & Maung (1971) for carcinoma of the uterus in several countries, especially England and Wales, Scotland, the Netherlands, Denmark, Norway, Sweden, New Zealand and Chile; but the intrusion was absent in the United States and Japan. They considered that 'the intrusion is related to change in the customary sexual relationships brought about by two world wars and their aftermath and, taken with other evidence, the pattern is best explained by an infective cause of the cancer'.

Higgins (1971) has compared recent mortality from cancer of the cervix uteri in the United States and the United Kingdom, pointing out that the increase in each 10-year age group did not occur after intervals of 10 years, and suggesting

Table 1. *Cancer of cervix deaths, England and Wales, 1951–70* (e_{ij})

(From the Registrar General's Statistical Review of England and Wales, Part I, 1951–70, Table 17.)

	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–74
1951–55	79	251	450	789	1123	1553	1750	1751	1621	1354
1956–60	62	283	615	920	1284	1467	1461	1608	1590	1427
1961–65	38	132	514	1097	1380	1530	1392	1548	1382	1313
1966–70	49	108	313	809	1424	1519	1591	1432	1403	1269

Table 2. *Female populations (thousands person-years)* (P_{ij})

(From the Registrar General's Statistical Review of England and Wales, Part II, 1951–70, Table A 2, home populations.)

	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–74
1951–55	77,740	83,220	78,240	84,850	82,670	76,620	68,710	61,450	53,090	43,110
1956–60	72,330	77,440	82,890	77,400	83,280	80,880	74,000	65,250	56,000	45,530
1961–65	71,916	72,653	77,292	81,919	77,048	81,942	78,294	70,020	59,736	48,174
1966–70	75,599	71,504	72,067	76,328	80,819	75,327	79,347	74,072	63,996	51,722

that the intrusions may be associated with changes in sexual behaviour which, however, were considered to affect different cohorts at different times. The difficulty of interpreting the patterns is increased by the possible effects of the introduction of screening programmes for cancer of the cervix, immigration, rising trends in hysterectomy, and changes in death certification.

METHOD AND RESULTS

In the present work the data have been analysed by a regression method similar to that used by Sacher (1960) in an application to mortality from tuberculosis, which in turn bears some similarity to earlier work (Kermack, McKendrick & McKinlay, 1934; Greenberg, Wright & Sheps, 1950; Spicer, 1954; Case, 1956).

The relevant data for mortality from cancer of the cervix in England and Wales, now including the quinquennium 1966–70, are shown in Tables 1 and 2.

Kermack *et al.* (1934) represented age-specific mortality rates from all causes as the product of two factors – one a function of age (x) alone, and the other a function of the year of birth alone:

$$q(x, t+x) = f(t)\, g(x), \tag{1}$$

where $f(t)$ is a function acting at the date of birth and $g(x)$ is the age function. They found that in England and Wales, and also in Scotland and to a lesser extent in Sweden, the relative mortality (i.e. the age-specific mortality as a percentage of the specific mortality for the same age group in a standard period such as 1845–55) depended primarily on the date of birth of the individuals concerned and only indirectly on the particular year under consideration.

Following Sacher, in the present analysis the rates of mortality from the

Table 3. *Derived factors in* (*a*) *particular solution*, (*b*) *transformed solution*

Age factors

	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–74
(*a*)	−7·34	−6·51	−6·00	−5·57	−5·40	−5·32	−5·45	−5·60	−5·81	−6·00
(*b*)	−7·34	−6·34	−5·62	−5·06	−4·67	−4·46	−4·43	−4·40	−4·44	−4·46

Time factors

	1951–5	1956–60	1961–5	1966–70
(*a*)	−0·552	−0·343	−0·171	0·000
(*b*)	−0·039	−0·002	0·000	0·000

Cohort factors

	1881	1886	1891	1896	1901	1906	1911	1916	1921	1926	1931	1936	1941
(*a*)	3·09	2·88	2·58	2·27	1·95	1·63	1·51	1·45	1·36	1·09	0·49	0·00	0·00
(*b*)	1·04	1·00	0·87	0·73	0·58	0·43	0·49	0·60	0·68	0·57	0·15	−0·17	0·00

particular cause of death are first transformed logarithmically to provide Gompertzians,

$$G_{ij} = \log (e_{ij}/P_{ij}), \tag{2}$$

where $i = 1, 2, \ldots, 10$ are the 5-year age classes, $j = 1, 2, 3, 4$ are the epochs of death (that is, the quinquennia for which the current data were obtained), and $k = 1, 2, \ldots, 13$ are the epochs of birth (from 1881 to 1941). In this case,

$$k = j - i + 10. \tag{3}$$

It seems best to work with the logarithms of the rates since the rates vary by several orders of magnitude between age groups. Accordingly, the following relation is postulated for the expectation of the Gompertzian:

$$E(G_{ij}) = \alpha_i + \beta_j + \gamma_k, \tag{4}$$

where α is the age factor, γ is the factor for influences operative in relation to the epoch of birth (called here the cohort factor), and β is the secular factor which operates in relation to the epoch of death (called here the time factor).

The following normal equations are derived from a 4×10 table of mortality rates, for least squared deviations of Gompertzians G_{ij}:

$$\alpha_i \sum_j w_{ij} + \sum_j w_{ij}\beta_j + \sum_j w_{ij}\gamma_k - \sum_j w_{ij}G_{ij} = 0 \quad (i = 1, 2, \ldots, 10), \tag{5}$$

$$\beta_j \sum_i w_{ij} + \sum_i w_{ij}\alpha_i + \sum_i w_{ij}\gamma_k - \sum_i w_{ij}G_{ij} = 0 \quad (j = 1, 2, 3, 4), \tag{6}$$

$$\gamma_k \sum_L w_{ij} + \sum_L w_{ij}\alpha_i + \sum_L w_{ij}\beta_j - \sum_L w_{ij}G_{ij} = 0 \quad (k = 1, 2, \ldots, 13), \tag{7}$$

where L is a variable orthogonal to k, so that $L = i + j - 1$. Thus in any one of the 13 equations (7), summation is for the fixed k, i.e. for all the range values of i and j such that $j - i = k - 10$.

Table 4. *Gompertzians: (a) observed, (b) fitted*

Age	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–74
1951–5										
(*a*)	−6·89	−5·80	−5·16	−4·68	−4·30	−3·90	−3·67	−3·56	−3·49	−3·46
(*b*)	−6·80	−5·69	−5·06	−4·61	−4·28	−3·92	−3·73	−3·56	−3·49	−3·46
1956–60										
(*a*)	−7·06	−5·61	−4·90	−4·43	−4·17	−4·01	−3·92	−3·70	−3·56	−3·46
(*b*)	−7·19	−5·76	−4·94	−4·46	−4·18	−4·03	−3·85	−3·67	−3·57	−3·46
1961–5										
(*a*)	−7·55	−6·31	−5·01	−4·31	−4·02	−3·98	−4·03	−3·81	−3·77	−3·60
(*b*)	−7·51	−6·12	−5·04	−4·38	−4·07	−3·97	−4·00	−3·82	−3·71	−3·58
1966–70										
(*a*)	−7·34	−6·49	−5·44	−4·55	−4·04	−3·90	−3·91	−3·95	−3·82	−3·71
(*b*)	−7·34	−6·51	−5·47	−4·48	−3·99	−3·86	−3·94	−3·97	−3·87	−3·73

The weights w_{ij} have been chosen to be inversely proportional to the sampling variances of the Gompertzians G_{ij}: that is,

$$\frac{1}{w_{ij}} \simeq \left(\frac{dG_{ij}}{de_{ij}}\right)^2 \text{var}\,(e_{ij}) \simeq \frac{1}{e_{ij}}, \tag{8}$$

assuming that the deaths in each group follow a Neyman Type A generalization of the Poisson distribution (with the extra parameter m common to all groups); and that the variances $e_{ij}(1+m)/P_{ij}^2$ of the rates are entirely due to the comparatively small number e_{ij} of deaths.

The program then computes the combinations of weights to be attached to each of the 27 factors according to the 27 normal equations. There are 10 factors for age, 4 for time and 13 for cohort, giving a 27×27 matrix of coefficients. The solution to the normal equations has 3 arbitrary constants, since the matrix of coefficients of the factors, though symmetrical and positive semi-definite, is singular and of nullity 3. A generalized inverse is obtained by means of a subroutine based on the method of Healy (1968). The program sets $\beta_4 = 0$, $\gamma_{13} = 0$ and $\gamma_{12} = 0$ in the particular solution provided. The conditions $\beta_4 = 0$ and $\gamma_{13} = 0$ present no difficulty as they correspond to datum levels relative to the most recent quinquennium of deaths (in 1966–70) and to the most recent cohort of women respectively. If desired, a constant k_1 can be added to the four time factors, and another constant k_2 to the 13 cohort factors, provided that $k_1 + k_2$ is subtracted from each age factor. This would not affect the overall form of the factors. The third condition arises as follows: an arbitrary linear trend can be added to the time factors, and the same linear trend to the age factors (beginning at ages 25–29, 1966–70), provided that the same trend is subtracted from the cohort factors. This has been done so as to transform $\gamma_{12} = 0$ into $\beta_3 = 0$, leaving $\beta_4 = 0$ and $\gamma_{13} = 0$ as before. The particular and transformed solutions are shown in Table 3.

In Table 4 are shown the observed and fitted values of G_{ij} ($G_{10,1}$ and $G_{1,4}$ are necessarily fitted exactly). The fitted values are for both the particular and the

transformed solution, as may be verified. In the observed and fitted values, the pattern falling with time is broken by the intrusion as described by Hill & Adelstein (1967), beginning with women aged 35–39 in 1956–60 and reaching women aged 55–59 in 1966–70. If, for the particular solution, corresponding values of G_{ij} are found before the addition of the time factors β_j, it appears that the pattern falls in every age group, whence it might seem that the intrusion in certain age groups could be regarded as due to the superimposition of the time factors on the age and cohort factors. That inference, however, would be incorrect, and stems from an unreasonably large positive trend in the time factors in the particular solution. Without further evidence, the most reasonable course seems to be instead to set this trend to zero in recent years, as in the transformed solution.

The fitted values in Table 4 were transformed to numbers of deaths in order to compare them with numbers of observed deaths. This test gave $\Sigma X = -1{\cdot}3$ and, significantly, $\Sigma X^2 = 72{\cdot}2$, with approximately 16 degrees of freedom, and no apparent pattern of individual deviations X. As the conditions strictly required for the use of a chi-square test are not present for these transformed values, the significance was checked by comparison with the theoretical distribution obtained from 12 Monte Carlo simulation runs, replacing the observed deaths in each cell by independent Poisson variables generated from them. This theoretical distribution has estimated mean values $\Sigma X^2 = 16$ and $\Sigma X = -0{\cdot}4$, a small bias. The test indicates that the deaths are more variable than a Poisson distribution of deaths would imply (or that we do not have the true populations at risk), but the hypothesis of a Neyman Type A distribution, with $m \doteq 3{\cdot}5$, is not rejected.

Two further analyses were performed in order to examine the robustness of the method. The first consists in making all the weights w_{ij} equal ($i = 1, \ldots, 10$; $j = 1, \ldots, 4$). This procedure gave factors for age ($-7{\cdot}34$, $-6{\cdot}52$, $-5{\cdot}95$, $-5{\cdot}55$, $-5{\cdot}32$, $-5{\cdot}28$, $-5{\cdot}42$, $-5{\cdot}56$, $-5{\cdot}77$, $-5{\cdot}97$), for time ($-0{\cdot}57$, $-0{\cdot}31$, $-0{\cdot}18$, $0{\cdot}00$) and for cohort (3·08, 2·83, 2·54, 2·23, 1·91, 1·59, 1·47, 1·41, 1·33, 1·09, 0·49, 0·00, 0·00), which differ very little from the particular solution above. The second analysis consists in omitting the time variable and regressing on ages and cohorts only. Again, the resulting factors for age ($-7{\cdot}34$, $-6{\cdot}33$, $-6{\cdot}00$, $-5{\cdot}03$, $-4{\cdot}63$, $-4{\cdot}41$, $-4{\cdot}37$, $-4{\cdot}33$, $-4{\cdot}36$, $-4{\cdot}36$) and cohort (0·90, 0·88, 0·77, 0·64, 0·50, 0·36, 0·43, 0·55, 0·65, 0·55, 0·13, $-0{\cdot}18$, 0·00) are fairly similar in form to the transformed solution above. The extent of agreement with results from these further analyses, to which the subsequent discussion equally applies, appears to provide some support for the present procedure, even if the method of choosing weights and populations at risk (which include single women) is not quite optimal.

DISCUSSION

In the transformed results the age factors rise steeply up to an age between 60 and 65 and then fall very slightly. A peak in incidence at ages 50–54 has, it is interesting to note, been found for cancer of the cervix in Norway (Breland,

1951), and peaks in morbidity in the same age group for Sweden for various periods of time between 1920 and 1949 (Lindell, 1952). Approximately 90% of deaths from cancer of the cervix occur within 5 years of the diagnosis (Bailar, 1964).

The cohort factors show decreases in successive cohorts, except for those born around 1941, 1916, 1921 and 1911, in order of magnitude of the increase from the cohort born 5 years earlier. Thus two groups of intrusions appear. One of these corresponds to that already described (Hill & Adelstein, 1967). The other refers to the most recent cohort for which data are available. But it will be necessary to look closely at data for 1971–5 to check the existence of the second intrusion, since it is based on rather few data and on an assumption about the time trend. A general decline in cohort factors may reflect a progressive environmental diminution of a carcinogenic agent (such as a virus to which the women are exposed) so that at important ages (or even *in utero*) females are exposed to less hazard, or fewer are exposed, than among their forerunners at the same ages. Provided that these ages are fairly constant and that their relative importance within cohorts does not change much, one is not restricted to an influence acting only at birth, or even at a single age, in order to use cohort factors. Important ages in this connexion may be ages at marriage or at first intercourse, considering the statistical link which has been reported between these ages and the frequency of the disease (Rotkin, 1967; Terris, Wilson, Smith & Nelson, 1967); also age at menopause (Kashgarian & Dunn, 1970).

A progressive change in the importance of different ages, such as that produced perhaps by exposure to a hazard at progressively earlier ages at marriage, or by increases in proportions married, or by changes in the age at menopause, occurring during this century, might be reflected chiefly in the cohort factors. Such an effect may contribute to the intrusions seen. On the other hand, any tendency towards more accurate or greater certification of deaths from cervical cancer between 1950 and 1970 should principally be represented in time factors. Its extent is thought to be small, but a ratio of only 0·85 has been reported for clinicians' to pathologists' diagnoses for deaths from this cause (Heasman & Lipworth, 1966). In the opposite direction should be the effect of a rising trend in the frequency of hysterectomy (Fairbairn & Acheson, 1969), and the effect on mortality, which has been questioned (Ahluwalia & Doll, 1968), of mass screening for carcinoma *in situ*. Misstatements of age are thought to contribute very little to the factors, but the grouping may warrant further investigation.

The present results are consistent with those of Hill & Adelstein (1967). A more definitive interpretation than that offered here, and greater discrimination between various models, may depend on the use of further data. It remains an open question how far it is proper to represent influences that may, for all we know, span much of a lifetime, by factors depending only on age at death, epoch of birth and epoch of death.

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Phage typing of coagulase-negative staphylococci and micrococci

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SUMMARY

A total of 3217 strains of coagulase-negative staphylococci and micrococci were tested for susceptibility to a collection of phages isolated from coagulase-negative cocci; it was concluded that a useful typing scheme could be developed. Of the strains of Baird-Parker's biotype 1, 72% were lysed by one or more phages, although rather a large proportion of strains are lysed by many phages to give a complex typing pattern.

Normal persons commonly yield 10 or more distinguishable strains of coagulase-negative cocci in cultures from the nose and the skin.

INTRODUCTION

During the last few years the coagulase-negative staphylococci (*Staphylococcus epidermidis* or *S. albus*) and micrococci have been the subject of several studies since it has become obvious that they can, in some circumstances, produce disease. In two conditions – endocarditis and bacteraemia originating in Spitz–Holter valves – it would be of particular value to have methods for type identification to enable studies of the origin of the infection to be pursued. We therefore set out to accumulate a collection of phages and to investigate their use for type identification. Shortly after we began work we found that Professor Winkler and Drs Verhoef and van Boven of Utrecht were similarly engaged and we have exchanged phages with them; some results from their studies have recently been published (Verhoef, van Boven & Winkler, 1971*a*, *b*, 1972).

MATERIALS AND METHODS

A total of 3217 strains of coagulase-negative, Gram-positive cocci were tested in our studies; 2413 were isolated from the nose and skin of hospital staff and patients and 804 from 'clinical' specimens. When allowance was made for repeat isolations from single individuals of strains having the same phage pattern and biotype (see below) a total of 1517 'independent' strains were available for analysis, and it is to these that all figures given in this paper refer.

All the strains were classified according to Baird-Parker's (1963) scheme, with the exception that, as suggested recently by Baird-Parker (1972, modifying Baird-

Table 1. *Biotypes of strains examined*

Biotype (Baird-Parker, 1965, modified 1972)	Subgroup (Baird-Parker, 1963)	Acid: Glucose Anaer.	Acid: Glucose Aer.	Acid: Mannitol Anaer.	Acid: Mannitol Aer.	Acid: Lactose	Production of: Phosphatase	Production of: Acetoin	No. of strains	
1	SII	+	+	−	−	(+)	+	+	832	972
	SV	+	+	−	−	+	−	+	140	
2	SIII	+	+	−	−	+	+	−	15	
3	SIV	+	+	−	−	−	−	+	38	
4	SVI	+	+	−	+	+	−	(+)	72	
*a**	(SI)*	+	+	+	+	+	+	+	39	
	M1	−	+	−	−	−	−	+	22	
	M2	−	+	−	−	+	−	+	43	
	M3	−	+	−	+	(+)	−	+	9	
	M4	−	+	−	+	+	−	+	10	
	M5	−	+	−	(+)	(+)	−	−	4	
	M6	−	+	−	+	+	+	−	5	
	M7	−	−	−	−	−	−	−	20	
	M unclass.	−	.	.	.	Various	.	.	187	
Unclassifiable		−	.	.	.	Various	.	.	81	
Total									1517	

* Strains with these characters are not included in Baird-Parker's (1972) classification and do not fall properly into his (1963) classification since his subgroup I strains were coagulase-positive.

(+) Variable reactions.

Table 2. *Characters of the typing phages*

Phage	Source	Propagating strain	Lytic pattern at 100 × RTD on the set of propagating strains: 15	27	RG	28	37	155	165	28A	A6C	A9C	71	275	48	82	456	157A	471A	459	275A
15	WFI	89	++	++	.	.	±	+	.	.	++	++	++	+	+	++	++	++	++	++	++
27	WFI	186	.	++	.	.	.	.	.	.	++	.	++	.	++	.	++	+	.	++	++
RG	Colindale	RG	.	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
28	WFI	170	.	.	.	++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
37	WFI	192	.	.	.	.	++	++	.	.	.	.	.	.	.	.	.	.	.	.	.
155	WFI	296	++	++	.	.	±	++	.	.	++	++	++	++	++	±	++	++	++	++	++
165	WFI	8	.	.	.	.	.	.	++	.	.	.	.	.	.	.	.	.	.	.	.
28A	WFI	SN14	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.
A6C	WFI	Deal 3	.	++	.	.	.	.	.	.	++	++	±	±	±	±	++	.	++	++	±
A9C	WFI	Howe 4	.	.	.	.	.	.	.	.	.	++	.	.	.	.	.	.	.	.	.
71*	Utrecht	240	+	++	±	.	±	++	+	±	++	++	++	++	++	±	++	+	++	++	++
275*	Utrecht	98	+	++	±	.	±	.	.	±	++	++	++	++	+	±	++	++	++	++	++
48*	Utrecht	81	.	+	.	.	.	.	.	.	.	.	++	.	++	±	.	+	.	.	++
82*	Utrecht	87	.	.	.	.	.	.	.	.	.	.	.	.	.	++	±	.	.	.	.
456	Utrecht	76	.	++	.	.	.	.	.	.	++	.	++	.	.	.	++	.	++	++	±
157A†*	Utrecht	382	.	.	.	.	.	.	.	.	.	.	±	.	.	.	.	++	.	.	±
471A*	Utrecht	330	.	++	±	.	±	.	.	.	++	±	.	+	.	±	++	.	++	++	±
459*	Utrecht	407	++	++	±	.	+	+	.	.	++	++	++	++	++	++	++	++	++	++	++
275A*	Utrecht	380	++	++	.	.	±	.	.	.	++	++	++	++	+	±	++	++	++	++	++

WFI = Wright–Fleming Institute. RTD = routine test dilution. ++ = >50 plaques – confluent lysis. + = 20–50 plaques. ± = <20 plaques.

* Strains included in the provisional typing set described by Verhoef *et al.* (1971*b*).

† Isolated from a biotype 4 strain.

Parker 1965), the term 'biotype' is used in place of 'subgroup' and biotype 1 is taken to include the strains previously classified as subgroup II and subgroup V. The tests used in our work are set out in Table 1. The methods used were substantially those of Baird-Parker but only a proportion of the strains were tested for fermentation of maltose. Tests for lactose fermentation and for phosphatase and acetoin production were performed as described by Baird-Parker (1963). Mannitol and glucose fermentation were examined by a modified method in media composed of (g/100 ml.): $NH_4H_2PO_4$ 0·1, KCl 0·02, $MgSO_4.7H_2O$ 0·02, yeast extract 1·0, tryptone 0·5, bromo-cresol purple 0·004, and agar 0·2. After sterilization, mannitol or glucose was added to a final concentration of 1·0%. In use, 10 ml. of the medium was inoculated with 0·5 ml. of an overnight broth culture and incubated for up to 5 days.

We observed 39 strains that were physiologically very similar to *Staphylococcus aureus* in that they fermented mannitol anaerobically, fermented lactose, and produced acetoin and phosphatase, but they all failed to produce a coagulase for human plasma and were insusceptible to the standard set of *S. aureus* typing phages; these are indicated in Table 1 as biotype *a* and are the same as the strains referred to by Corse & Williams (1968) as *S. albus*.

Phages were isolated by 'cross-culture', spotting the supernatant fluid from 6 hr. cultures onto a plate spread with a culture of similar age. After overnight incubation plaques were picked and the phages purified by subculture; one plaque was suspended in 0·5 ml. broth, which was immediately spotted on a plate flooded with a 6 hr. culture of the susceptible strain. After two single-plaque subcultures the phages were propagated in infusion broth (Southern Group Laboratories) containing 0·04% $CaCl_2$, filtered through Millipore (HAWP) membranes having a 0·45 μm. pore size, and preserved at +4°C.

The solid medium used for titration of phages and phage typing was formulated for us by Oxoid Laboratories. It contained 1·25% Peptone L 37 (Oxoid), 0·75% Lab-Lemco powder, 0·7% Agar Number 1 (Oxoid) and 0·04% calcium chloride.

All strains were tested with phages used at a titre of 100 × RTD (see Blair & Williams, 1961) and almost all untypable strains were retested with undiluted phage filtrates (mostly 1000 or 10,000 × RTD). The term 'typable' is used for strains showing lysis with any phage used at 100 × RTD or stronger.

A total of 13 phages were isolated and tested, and a further 14 were received from Professor Winkler and his colleagues. After testing 2894 strains with this set of 27 phages, the results were analysed and eight phages found to be redundant; these were discarded from the typing set.

The results in this paper are based on the set of 19 phages listed and characterized in Table 2.

RESULTS

Of the total collection of 1517 strains, 591 (39%) showed strong (++) lysis by one or more of the phages used at 100 × RTD, and a further 154 (10%) showed weak lysis. Of the untypable strains, 110 (7% of the total) were lysed when the stronger phage filtrates were used giving a total of 855 (56%) of typable strains.

Table 3. *Phage sensitivity of strains of different biotypes*

	No. of strains tested	% 'typable'*	No. 'typable' Short patterns†	No. 'typable' 'Long' patterns†
Staphylococcus				
Biotype 1	972	72	480	222
Biotype 2	15	27	4	0
Biotype 3	38	50	18	1
Biotype 4	72	45	31	1
Biotype *a*	39	56	19	3
Total 'staphylococcus'	1136	68	552	227
Micrococcus				
Biotype M 1	22	14	3	0
Biotype M 2	43	9	3	1
Biotype M 3	9	22	2	0
Biotype M 4–6	19	–	0	0
Biotype M 7	20	15	3	0
Unclassified micrococci	187	19	32	3
Total 'micrococcus'	300	16	43	4
Unclassifiable	81	36	26	3

* Typable = any pattern at RTD × 100 or stronger.
† 'Long pattern' = reactions with six or more phages (see text).

Table 4. *Number of reactions in lytic patterns*

No. of + + reactions	No. of strains	% of total strains giving + + reactions
1	239	40
2	57	10
3	34	6
4	34	6
5	17	3
6	15	3
7–9	93	16
10–12	84	14
13 or more	18	3
Weak reactions only	264*	
No reaction	662	
Total	1517	

NOTE. The 'long' pattern ordinarily comprises 6–13 lytic reactions, but not all of them are classed as + +.

* 154 gave weak (+) reactions with phage at RTD × 100 and 110 were typable with phage used at a higher concentration.

Table 5. *Phage patterns represented by ten or more cultures*

Lytic pattern	Total	Staphylococcus biotype			Micrococci	Unclassified
		1	2, 3, 4	*a*		
'Long' (i.e. reactions with 6 or more phages)	234	222	2	3	4	3
71	20	14	0	3	0	3
82	23	18	2	2	0	1
82 + several weak reactions	54	52	0	0	0	2
37	56	8	20	1	21	6
275A	19	15	1	1	2	0
157A	43	38	1	2	0	2
456; 456/459; 459	28	27	1	0	0	0
RG	24	14	2	0	5	3
28	12	12	0	0	0	0
A6C/A9C	96	87	2	1	4	2
15	10	5	0	3	1	1

The proportion of typable strains varied substantially between the different biotypes (Table 3); 72% of strains of biotype 1 were sensitive compared with 44% of the strains in biotypes 2, 3 and 4 and 16% of the micrococci. About half (56%) the strains in the *S. aureus*-like biotype *a* were sensitive. These differences probably reflect the fact that most of the phages were isolated from strains of biotype 1.

Most of the strains of staphylococci and micrococci were lysed by several different phages to give pattern reactions, as in the *S. aureus* typing system. A rather serious drawback, at present, is the fact that some 36% of the strains that are lysed by any phage are lysed by six or more phages and 17% were lysed by ten or more (Table 4). Not all these 'long patterns' are identical but the differences among them do not seem to be consistent enough to permit their subdivision into distinguishable patterns. The 'long' patterns were proportionately more common among strains of biotype 1 than among the relatively few typable strains of other biotypes (Table 3).

Table 5 sets out the distribution over the biotypes of strains belonging to phage 'types' represented by ten or more strains, and also the strains giving the 'long' pattern. All but one of the types were seen in strains of more than one biotype, although with the exception of lysis with phage 37, which was seen rather frequently among the micrococci, there was no segregation of particular patterns into particular biotypes.

Reproducibility

We typed a large number of strains received from Dr R. J. Holt, who had isolated them from patients with chronic bacteraemia complicating Spitz-Holter valves. Like Verhoef *et al.* (1972), who also examined some of these strains, we obtained either identical typing patterns from all the strains isolated from individual patients, or in some cases two or more sets of identical patterns.

We also examined the reproducibility of repeat tests on single strains examined

Table 6. *Percentage distribution of various phage patterns among staphylococci and micrococci from various sources*

Phage pattern	Source of strains: Carrier sites	Wounds	Blood culture	Urine
'Long'	16	15	26	4
71 + or − other reactions	3	1	.	.
82 + or − other reactions	6	6	3	3
37	3	11	5	6
275A	1	.	.	.
157A	3	.	2	.
456; 459; 456/459	2	1	7	1
RG	2	1	3	.
28	.	.	1	2
A6C/A9C	6	8	9	6
15	1	.	.	.
Others	13	14	6	5
N.T.	43	40	35	75
Total no. of strains	1132	84	86	107

N.T. = not typable.

on one day and after an interval and our results conformed with those of Verhoef *et al.* (1972); duplicate tests on the same day gave loss or gain of only one + + reaction in one of 17 tests, and in duplicate tests on separate days at 3 months' interval 2 of 10 strains showed loss or gain of 1 + + reaction and 1 strain 4 + + differences. In these tests the strains examined all showed at least 7 + + reactions.

Types in relation to source

Table 6 summarizes the distribution of the commoner types in carrier sites and pathological lesions. There are no striking differences between the strains from carrier sites and those from wounds or blood cultures; a large proportion of the strains from urine proved to be untypable.

Numbers of distinguishable types on carriers

A number of members of the laboratory staff had swabs taken from the nose and from six skin sites on two or more occasions. The swabs were inoculated on nutrient agar plates and incubated overnight. Up to five colonies of each distinguishable colony type were chosen at random and subcultured for phage-typing and biotyping. Strains were regarded as different if they were of a different biotype (even if, as rarely occurred, they had the same phage pattern) or if, regardless of biotype, the phage pattern differed significantly; for this purpose differences in typing patterns were interpreted in the way used in *S. aureus* typing (Blair & Williams, 1961). For this analysis, all untypable cultures were counted as one strain.

Table 7. *Numbers of distinguishable strains in cultures from nose and skin*

No. of distinguishable strains	No. of cultures contributing colonies																								
	13	15	11	8	8	11	7	5	7	9	13	6	3	3	7	4	2	1	0	2	0	0	3	1	2
11	.	.	.	.	.	.	.	.	.	.	1	1	1	.	.	1	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	.	.	.	1	.	.
9	.	.	.	.	.	.	.	.	.	1	2	1	.	1	2	1	.	.	.	.	.	.	.	.	2
8	.	.	.	.	.	.	.	.	.	2	2	.	.	.	1	1	.	.	.	.	.	.	1	.	.
7	.	.	.	.	1	1	3	1	1	.	3	.	.	1	1	.	1	.	.	.	.	.	1	.	.
6	.	.	2	2	.	2	.	.	1	2	2	2	.	.	.	.	1	1	.	1	.	.	.	.	.
5	.	3	.	1	4	1	2	2	2	1	1	1	1	.	2	.	.	.	.	1	.	.	.	1	.
4	4	5	4	1	2	2	2	.	2	2	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.
3	4	4	1	3	1	2	.	.	1	1	1	1	1	.	.	.	.	.	.	.	.	.	.	.	.
2	3	2	3	.	.	2	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1	2	1	1	1	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28

No. of colonies tested from each sample

The numbers of colonies examined from the different subjects varied between 2 and 125. Table 7 illustrates the results from 90 swab cultures, from each of which between 4 and 28 colonies were tested; the numerals in the body of the figure are the number of cultures yielding the indicated number of distinguishable types. It will be seen that when more than 10 different colonies were tested, about 7–11 strains could generally be distinguished in each culture. There were also five subjects who, from a total of 16 swabs, each yielded more than 90 colonies; they had between 25 and 45 distinguishable strains.

The same study showed that individuals commonly harbour staphylococci having the same phage pattern over periods at least up to 16 weeks; in 12 of the 19 persons in whom prolonged carriage was observed, the strain concerned belonged to biotype 1 and showed the 'long' phage pattern.

DISCUSSION

The preliminary studies reported here, in confirmation of the work reported by Verhoef *et al.* (1971*a*, *b*, 1972), demonstrate the practicability of bacteriophage type-identification of the coagulase-negative staphylococci. Most of the strains for which some pathogenic role is postulated fall into Baird-Parker's biotype 1 (previously known as Subgroups II and V) and in our experience isolation of phages for strains of biotype 1 has been easier than for other biotypes or for micrococci. We have not found any advantage from the use of Mitomycin C (Verhoef *et al.* 1971*a*) as an inducing agent for the isolation of phages.

We have included 19 phages in our provisional typing set; this includes 8 of the 18 phages in the set proposed by Verhoef *et al.* (1971*b*), one other phage received from these workers but not included in their set, 10 phages isolated by us, and 1 phage from a culture collection. This set was selected from a larger collection after testing nearly 3000 strains of staphylococci and micrococci. With it useful lytic reactions can be obtained with about 72% of biotype-1 *S. epidermidis*. The main practical difficulty has been the rather large proportion of strains that are very sensitive to the phages and are lysed by ten or more different phages.

The reproducibility of the reactions, both in repeated tests of single isolates, and in repeated isolates from infected patients, seems to be quite adequate, although there is certainly variability among the strains with the 'long' typing pattern; distinct types within this group have not yet been recognized but may well exist.

Normal persons evidently carry quite large numbers of distinguishable strains of staphylococci on their body. If any attempt is to be made to seek the origins of post-operative wound infections by pre-operative examinations, it will therefore be necessary to type a considerable number of isolates.

There is not, from our work, any clear indication that particular phage patterns within biotype 1 characterize strains having special pathogenic properties, but our experience is not yet sufficient for a clear statement on this point.

Since coagulase-negative staphylococci are commonly resistant to several antibiotics, we wondered whether resistance could be conveyed from them to

coagulase-positive *S. aureus* by transduction. So far we have been quite unsuccessful in demonstrating this.

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Examination of two bacterial strains designated '*Brucella suis* biotype 5'

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SUMMARY

The morphological, cultural, biochemical, serological and pathogenic properties of two bacterial strains of the group designated '*Br. suis* biotype 5' were examined. Both strains were found to be atypical of the genus *Brucella* in many of these characteristics. No serological relationship to known brucella strains could be detected. On the basis of the evidence obtained the two strains examined were classified as *Moraxella duplex* and the status of '*Br. suis* biotype 5' questioned.

INTRODUCTION

Renoux & Philippon (1969) proposed that certain organisms isolated from the reproductive tracts of cattle and sheep and from abortion material should be included in the classification scheme for the genus *Brucella* as examples of a fifth biotype of *Brucella suis*. These organisms differed from other *Br. suis* strains in their resistance to Safranine O and in their oxidative metabolic pattern. According to Renoux & Philippon (1969) all isolates were rough and thus could not be characterized by phage typing nor by agglutination reactions with mono-specific antisera to *Br. abortus* and *Br. melitensis*. Recently the opportunity occurred to examine two of the isolates of Renoux and Philippon and the results obtained indicated that the inclusion of these strains in a new brucella biotype should be re-examined.

MATERIALS AND METHODS

Bacterial strains

The two strains designated '*Br. suis* biotype 5' were provided by Dr A. Philippon of I.N.R.A., Station de Pathologie de la Reproduction, Nouzilly, France, as freeze-dried cultures B58 and 4607. Strain B58 was of bovine origin and strain 4607 was of ovine origin. Both were studied by Renoux & Philippon (1969) and some of their characteristics reported in their published results. Brucella and other bacterial strains used as controls in this study were from the culture collection maintained at this laboratory.

Morphological examination

The general morphology of heat-fixed organisms was studied in smears stained by Gram's method or with Loeffler's methylene blue. Wayson's method was used for demonstrating bipolar staining, the Ziehl-Neelsen method and the modified Köster's method (Christoffersen & Ottosen, 1941) for detecting acid-fast staining and the method of Lechtman, Bartholomew, Phillips & Russo (1965) for demonstrating spores. Flagella staining was done according to Leifson (1951) and capsule staining according to Howie & Kirkpatrick (1934). Negative staining with indian ink was done according to Duguid (1951). Electron-microscopic examination of negatively stained preparations was done as described by Corbel & Phillip (1972).

Bacteriological examination

Standard bacteriological procedures for the identification of bacteria were used (Cowan & Steel, 1965). Identification of non-fermentative organisms was done using the medium described by Sellers (1964). Sterile 50% (w/v) lactose was added to some tubes in place of D(+) glucose. The methods used for the typing of *Brucella* species were as recommended by Morgan & Gower (1966) and Alton & Jones (1967). Measurements of oxidative metabolic rate with various substrates were made using a Gilson differential respirometer.

Phage sensitivity tests

Bacterial strains were tested for susceptibility to lysis by the Tbilisi brucella-phage and by the phages A422, M51 and S708 of Moreira-Jacob (1968). Phage suspensions were used at routine test dilution (RTD $\simeq 5 \times 10^4$ p.f.u./ml.) and 10,000 × routine test dilution (10,000 RTD $\simeq 5 \times 10$ p.f.u./ml.) according to procedures recommended by Alton & Jones (1967).

Antibiotic sensitivity determination

This was done by the multiple disk method using confluent growths of the organism on trypticase soy agar. Zones of inhibition were measured after 24 hr. incubation at 37°C. Multodiscs (Oxoid, London) were used for the assays.

Serological examination

Rabbit and bovine antisera to *Brucella* spp. and *Y. enterocolitica* IX were prepared according to Corbel & Cullen (1970). Antisera to *Francisella tularensis* and *Mima polymorpha* were obtained from Difco Laboratories, Detroit.

The serum agglutination test (SAT), the complement fixation test (CFT), the Rose Bengal plate test and disulphide reduction and antiglobulin tests were done by procedures described by Morgan *et al.* (1971). Immunodiffusion and indirect immunofluorescence tests were performed on extracts of ultrasonically disrupted organisms according to Corbel & Cullen (1970).

Agglutination tests for antibodies to rough organisms were done according to Diaz, Jones & Wilson (1967).

The serological response of animals to inoculation with B58 and 4607 was examined by intramuscular injection of guinea-pigs with doses of *ca.* 10^{11} heat-killed organisms and intravenous injection of rabbits with 5×10^{10} heat-killed organisms. The responses of animals receiving live organisms by the intraperitoneal and subcutaneous routes were also examined. Serum samples were tested for antibodies to smooth and rough brucella strains using *Br. abortus* strain 99, *Br. canis* RM 6-66 and *Br. ovis* as antigens. Antibodies to the homologous bacterial strains were also detected by agglutination, CFT and precipitin tests using suspensions of B58 and 4607 as antigens. The anamnestic response to brucella antigens was examined by intravenous injection of rabbits inoculated with *Br. abortus* strain 19 some 6 months previously, with suspensions of B58, 4607 and *Br. abortus* 45/20 organisms standardized turbidimetrically to contain *ca.* 10^{10} organisms/ml. Blood samples were collected daily for 7 days after injection and twice weekly thereafter.

Determination of virulence

Virulence was assessed by intravenous inoculation of pairs of adult rabbits with *ca.* 10^{11} viable B58 or 4607 organisms. Male and female weaned albino guinea-pigs were injected by the intraperitoneal route with doses of 5×10^{11} viable organisms. Six female guinea-pigs were given similar doses of these organisms at about the fortieth day of pregnancy. Three of these animals were killed and examined 3 weeks after inoculation and the other three were allowed to proceed to parturition before killing and autopsy of themselves and their offspring. All animals were examined *post mortem* for macroscopic signs of disease and in addition smears were made of the viscera for microscopic examination. The spleens of all animals were emulsified and cultured on sheep blood agar, serum dextrose agar, MacConkey bile salt agar and Levine eosin-methylene blue agar for up to 14 days at 37° C. Fetuses and membranes recovered from pregnant animals were treated similarly.

Albino mice of *ca* 30 g. weight were inoculated intraperitoneally with *ca.* 5×10^{10} viable B58 or 4607 organisms. Six similar mice were also inoculated by the intracerebral route with *ca.* 10^7 viable organisms. All mice were killed 14 days after inoculation and smears and cultures prepared from the internal organs, including the brain.

RESULTS

Morphological examination

Gram-stained smears of B58 and 4607 showed small Gram-negative cocci or cocco-bacilli arranged mainly in pairs or short chains with a proportion of single cells. Methylene blue stained preparations showed mainly diplococcal forms. No indication of bipolar staining was evident in smears stained by Wayson's method and no spores or flagella were observed in preparations stained by the relevant method. The organisms were not acid-fast and stained poorly by Köster's method. No capsules could be seen in indian ink preparations but were visible in preparations made according to Howie & Kirkpatrick (1934) as stained areas surrounding

the bacterial cells (Pl. 1, figs. 1–4). Diplococcal and chain formations were also readily demonstrated by this method which does not involve heat fixation. Electron-microscopic examination confirmed these results and the capsules were visible as distinct layers surrounding the bacterial cells. The pairs of cells were 1·25–1·45 μm. in length by 0·6–0·70 μm. in width with capsules 0·15–0·17 μm. thick for the B58 strain. For the 4607 strain the pairs of cells were 1·15–1·25 μm. in length, 0·50–0·72 μm. in breadth and with capsules 0·10–0·30 μm. thick (Pl. 2, figs. 1, 2). Because of the capsules the phosphotungstic acid stain penetrated poorly into the surface of the organisms and subcapsular structures could not be visualized. It was difficult in most cases to distinguish the intersections between diplococci. No capsules were observed in preparations of smooth and rough brucella strains stained and examined by the same methods.

Bacteriological examination

Cultural characteristics

Both strains grew on nutrient agar, serum dextrose agar, trypticase soy agar and Albimi brucella agar, producing visible growth in 24 hr. at 37° C. Growth also occurred, but more slowly, at 20° C. No growth occurred at 4° or 50° C. The growth of both strains was enhanced on serum-containing media. On all media strain B58 grew more rapidly than 4607. Initially colonies on these media resembled morphologically those of brucella strains, but differed in their more rapid growth rate. On prolonged incubation, however (6–7 days at 37° C., 10 days at 20° C.) large umbonate colonies, 7–8 mm. in diameter for the B58 strain and 5–6 mm. in diameter for the 4607 strain, were produced.

On sheep, horse and rabbit blood agar both strains produced small white hemispherical colonies in 24 hr. at 37° C. No haemolysis was observed. Similar growth was produced on lysed and heated blood media.

Both strains also produced colonies on MacConkey bile salt agar, deoxycholate citrate agar, Levine eosin–methylene blue agar and sheep blood thioglycollate agar. The colonies were lactose-negative and on bile salt media similar in appearance to the growth on nutrient agar. On eosin–methylene blue agar lavender-coloured colonies with a transparent entire margin were produced in 2–3 days at 37° C. B58 also grew on Wilson and Blair medium producing transparent drop-like colonies 0·1 mm. in diameter after 7 days incubation, but 4607 did not grow on this medium. Neither strain grew anaerobically and growth was neither enhanced nor inhibited in the presence of added CO_2. Both strains grew readily in the presence of the brucella dyes, basic fuchsin at 1/50,000, thionin at 1/50,000, pyronin Y at 1/100,000, safranin O at 1/5000 and thionin blue at 1/100,000. B58 grew well in the presence of 1/50,000 methyl violet but 4607 did not grow in the presence of this dye. *meso*-Erythritol at concentrations of 10 mg./ml. did not significantly affect growth. B58 grew readily on serum dextrose agar containing 10 units of penicillin G per ml. but 4607 was inhibited on this medium. Both strains grew readily on the serum dextrose agar antibiotic medium of Kuzdas & Morse (1956). In semi-solid thioglycollate media both strains produced a uniform disk of growth in the aerobic layers and no growth in the deeper layers.

Table 1. *Biochemical reactions of strains B*58 *and* 4607

Test	B58	4607
H_2S	−	−
Urea	+ (8 hr.), + + + (24 hr.)	− (8 hr.) − (24 hr.) − (21 days)
KCN	+	−
Methyl Red	−	−
Voges Proskauer	−	−
Indole	−	−
Nitrate reduction	−	−
Gelatin stab	Surface growth. No liquefaction in 21 days	Surface growth. No liquefaction in 21 days
Litmus milk	No acid or digestion. Reduction at 21 days	No change
Loeffler's serum slope	Growth. No liquefaction	Growth. No liquefaction
Brewer's thioglycollate medium	Discoid surface growth	Discoid surface growth
Catalase	+	+
Oxidase	+	+
Motility 37° C.	−	−
Motility 20° C.	−	−
Decarboxylase, arginine	−	−
Decarboxylase, lysine	−	−
Decarboxylase, ornithine	−	−
Anaerobic growth	−	−
Microaerophilic growth (10 % CO_2)	+	+

Biochemical properties

Both B58 and 4607 showed very limited biochemical activity in the conventional tests as shown in Table 1. Strain B58 possessed strong urease activity but 4607 had no urease activity. Neither strain produced H_2S detectable with lead acetate papers or on Kligler's medium. Both strains showed very limited fermentative activity towards carbohydrates in peptone water (Table 2), but in Hugh and Leifson's medium acid was very slowly produced from D(+)-glucose. Both strains grew on Seller's medium, without production of gas, fluorescent pigment or substantial fermentation of sugars. B58 slowly released acid from D(+)-glucose on this medium although 4607 did not. In the presence of lactose, B58 produced an alkaline slant but 4607 produced no change. Both strains reacted positively in tests for catalase and oxidase. In oxidative metabolism tests conducted in the Gilson differential respirometer, both strains oxidized a number of substrates including arginine, dextrose, asparagine and ribose.

Where the cultural and biochemical tests performed coincided with those done by Renoux & Philippon (1969), the results obtained were generally consistent with those reported.

Phage susceptibility

B58 and 4607 were both resistant to lysis by the four brucella phages tested. Smooth cultures of B58 were used for the test and these were quite refractory to lysis by any phage at RTD or 10,000 RTD, including the *Br. suis* phage S708.

Table 2. *Carbohydrate reactions of strains B58 and 4607*

	B58 Day number						4607 Day number					
Substrate	1	3	7	10	14	21	1	3	7	10	14	21
Adonitol	–	–	–	–	(+)	+	–	–	–	(+)	+	+
Aesculin	–	+	+	+	+	+	–	–	–	–	–	–
Amygdalin	–	(+)	+	+	+	+	–	(+)	+	+	+	+
Arabinose	–	–	–	–	–	–	–	–	–	–	–	–
Cellobiose	–	(+)	+	+	+	+	–	(+)	+	+	+	+
Dextrin	–	–	–	–	–	–	–	–	–	–	–	–
Dulcitol	–	–	–	–	–	–	–	–	–	–	(+)	+
Erythritol	–	(+)	+	+	+	+	–	(+)	+	+	+	+
Fructose	–	–	–	–	–	–	–	–	–	–	–	–
Glucose	–	–	–	–	–	–	–	(+)	(+)	+	+	+
Glycerol	–	–	–	–	–	(+)	–	–	–	–	–	–
Glycogen	–	–	–	–	(+)	(+)	–	–	–	–	(+)	+
Inositol	–	–	–	–	–	–	–	–	–	–	–	–
Inulin	–	–	–	–	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–	–	–	–	–
Maltose	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	(+)	+	–	–	–	–	(+)	+
Mannose	–	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	(+)	+	+	+	+	–	(+)	+	+	+	+
Raffinose	–	–	(+)	+	+	+	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	–	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	(+)	–
Sucrose	–	–	–	–	–	–	–	–	–	–	–	–
Trehalose	–	–	–	–	–	–	–	–	–	–	–	–
Xylose	–	–	–	–	–	–	–	–	–	–	–	–
Seller's lactose	ALK	ALK	ALK	ALK	ALK	ALK	(A)	(A)	(A)	(A)	A	A
Seller's glucose	–	(A)	A	A	A	A	–	(A)	(A)	A	A	A
Seller's aerogenesis	–	–	–	–	–	–	–	–	–	–	–	–
Seller's anaerobiosis	–	–	–	–	–	–	–	–	–	–	–	–
Seller's pigment	–	–	–	–	–	–	–	–	–	–	–	–
Hugh & Leifson's O/F medium	–	–	(F)	F	F	F	–	–	(F)	F	F	F
Kligler's medium	–	ALK	ALK	ALK	ALK	ALK	–	–	–	–	(A)	(A)
Kligler's aerogenesis	–	–	–	–	–	–	–	–	–	–	–	–
Kligler's H_2S	–	–	–	–	–	–	–	–	–	–	–	–
Koser's citrate	–	–	–	–	–	–	–	–	–	–	–	–
ONPG	–	–	–	–	–	–	–	–	–	–	–	–

– = no change, + = acid production, F = fermentation, O = oxidation, () = slight reaction, A = acid, ALK = alkali.

Table 3. *Antibiotic sensitivity of strains B58 and* 4607

Antibiotic	Concentration	Strain B58	Strain 4607
Ampicillin	2 u.	R	S
Ampicillin	25 u.	S	S
Bacitracin	25 u.	R	R
Chloramphenicol	10 μg.	S	S
Chloramphenicol	50 μg.	S	S
Chlortetracycline	25 μg.	S	S
Chlortetracycline	50 μg.	S	S
Erythromycin	15 μg.	S	S
Furazolidone	15 μg.	R	R
Furazolidone	50 μg.	R	R
Kanamycin	30 μg.	S	S
Mitomycin C	1 μg.	R	R
Mitomycin C	5 μg.	R	R
Neomycin	10 μg.	S	S
Neomycin	30 μg.	S	S
Novobiocin	30 μg.	S	S
Oxytetracycline	30 μg.	S	S
Penicillin G	5 u.	R	S
Penicillin G	10 u.	R	S
Polymyxin B	10 u.	R	R
Streptomycin	10 μg.	R	R
Streptomycin	25 μg.	R	R
Tetracycline	10 μg.	S	S
Tetracycline	50 μg.	S	S
Trimethoprim + sulphamethoxizole	25 μg.	R	R
Triple sulphonamides	50 μg.	R	R
Triple sulphonamides	300 μg.	R	R
Spectinomycin	25 μg.	S	S
Amphotericin B	10 μg.	R	R

R = Not inhibited. S = Inhibited.

Strain 4607 was only available as a rough strain and this was also resistant to the phages at all concentrations tested.

Antibiotic sensitivity

Both strains were resistant to sulphonamides, furazolidone, and trimethoprim even in high concentrations. They were also resistant to streptomycin, polymyxin B and bacitracin but both were sensitive to chloramphenicol, tetracyclines, kanamycin, neomycin, erythromycin, novobiocin and ampicillin. B58 was resistant to penicillin G and only moderately sensitive to ampicillin whereas 4607 was highly sensitive to both antibiotics (Table 3).

Pathogenicity

No evidence of any pathological disturbance was obtained after inoculation of rabbits, mice and guinea-pigs with large numbers of viable B58 or 4607 organisms. Even intracerebral inoculation of mice failed to produce signs of disease. Pregnant

Table 4. *Effect of strains B58 and 4607 on the anamnestic response to* Br. abortus *antigens*

Animal*	Inoculum	SAT	2ME	Quantitative RBPT	CFT	Immunodiffusion lps	Immunodiffusion ssa	
BS 1	45/20	1/20	1/20	1/2	1/20	0	2	Day 0
BS 2	45/20	4/10	3/10	1/2	1/10	0	2	
BS 3	B58	1/10	1/10	1/1	1/4	0	1+	
BS 4	B58	1/20	4/10	—/1	1/4	0	1+	
BS 5	4607	1/10	1/10	—/1	1/4	0	1+	
BS 6	4607	2/20	3/20	1/2	1/10	0	2	
BS 1	45/20	1/80	3/20	1/8	1/40	0	2	Day 4
BS 2	45/20	3/40	4/20	1/4	1/20	0	2	
BS 3	B58	1/10	1/10	1/1	1/4	0	1+	
BS 4	B58	2/20	4/10	—/1	1/4	0	1+	
BS 5	4607	1/10	—/10	—/1	1/4	0	1+	
BS 6	4607	3/20	2/20	1/2	1/10	0	2	
BS 1	45/20	4/320	3/80	1/32	1/200	1	2+	Day 7
BS 2	45/20	1/320	4/40	1/16	1/200	1	2+	
BS 3	B58	1/10	1/10	1/1	1/4	0	1+	
BS 4	B58	2/20	2/10	—/1	1/4	0	1+	
BS 5	4607	—/10	—/10	—/1	1/4	0	1	
BS 6	4607	3/20	3/20	1/2	1/10	0	2	

2ME = 2-mercapto-ethanol reduction test.
RBPT = Rose Bengal plate test.
lps = lipopolysaccharide antigen of *Br. abortus*.
ssa = sub-surface antigens of *Br. abortus*.
* Rabbits BS 1–6 had been inoculated with *Br. abortus* strain 19 *ca.* 6 months prior to this experiment.

guinea-pigs inoculated at about mid-term did not show evidence of disturbance and they eventually gave birth to healthy offspring. No organisms were recovered *post mortem* from any animal inoculated with these strains and no microscopical evidence was obtained of infection. It must be concluded that these two strains have a very low pathogenic potential for laboratory animals.

Serological properties

On emulsifying suspensions of organisms in 1/1000 acriflavine B58 was found to react as a smooth organism and was not agglutinated, whereas 4607 was immediately agglutinated and hence rough. B58 formed stable suspensions in 0·15 M-NaCl but 4607 auto-agglutinated in this medium. Both organisms were readily agglutinated by many samples of 'normal' rabbit and bovine sera as well as antisera to a variety of Gram-negative and Gram-positive organisms.

No reaction was obtained in CF tests with antisera to *Br. abortus* 544, *Br. abortus* 45/20, *Br. ovis* or *Br. canis* RM 6–66.

Neither B58 nor 4607 absorbed antibodies to rough or smooth brucellas from anti-*Brucella* sera. Similarly extracts of disrupted B58 or 4607 organisms did not react in immunodiffusion tests with antisera to *Brucella* spp. (Pl. 3, fig. 1) although extensive cross-reactions were observed when these sera were tested against

extracts of various brucella strains. In immunodiffusion tests with homologous antisera, 4607 and B58 showed extensive cross-reactions with each other but not with brucellas (Pl. 3, fig. 2).

Attempts were made to detect low concentrations of masked antigens cross-reacting with brucellas by provoking an anamnestic response to brucella antigens by inoculating *Br. abortus* sensitized rabbits with B58 and 4607 organisms. No evidence of any increase in titre of antibody to *Br. abortus* was detected in these animals although *Br. abortus* 45/20 produced rapid increases in titre within 4–5 days when inoculated into similar animals. That this was anamnestic and not a primary response was shown by the substantial titres of reduction-stable agglutinins in these sera (Table 4).

Extracts of both B58 and 4607 produced precipitin lines on diffusion against polyvalent antiserum to *Mima polymorpha*. A reaction of identity was given by both strains on diffusion against this antiserum which produced no reaction on diffusion against extracts of *Brucella* spp. (Pl. 3, fig. 3). This serum contained antibodies to both *Moraxella* spp. and *Acinetobacter* spp. (*Herellea vaginicola* and *M. polymorpha* respectively). Strains B58 and 4607 produced reactions of identity with the *Moraxella duplex* reference strain but no cross-reaction with *Acinetobacter* spp. (Pl. 4, fig. 4).

These results were confirmed by indirect immunofluorescence and complement fixation tests.

DISCUSSION

The morphological characteristics of the two bacterial strains examined were distinct from those typical of the *Brucella* genus. Thus the growth of most organisms in pairs or short chains and the presence of discrete capsules are not features generally considered typical of brucellas. The lack of acid-fast staining with Köster's stain and the tendency to retain crystal violet with Gram's stain also distinguished these organisms from typical brucellas.

The electron-microscopy results confirmed the presence of capsules and negatively stained preparations were clearly quite different from typical *Brucella* strains which normally show a rugose cell surface with no evidence of capsule formation.

The cultural characteristics of the two strains B58 and 4607 emphasized their differences from typical brucella strains. Their rapid growth on a wide range of media at 20° and 37°C. suggested that B58 and 4607 were not brucellas, and their ability to grow on selective media such as MacConkey, deoxycholate-citrate, Levine eosin–methylene blue agar and, in the case of B58, on Wilson and Blair medium, confirmed this. Furthermore the ability to grow in the presence of safranine O, lack of H_2S production and of nitrate-reducing activity, and, in the case of 4607, absence of urease activity, are all inconsistent with the *Br. suis* group. It should be mentioned, however, that 10 out of the 13 strains studied by Renoux & Philippon (1969) were H_2S producers and in this respect B58 and 4607 were not representative of the group.

Minimal fermentative activity towards carbohydrates, although a property of

the *Brucella* genus, with the exception of *Br. neotomae*, is by no means exclusive to this group and is in fact typical of the tribe Mimeae (de Bord, 1942).

Similarly the oxidative metabolic pattern, although consistent with that described by Renoux & Philippon (1969) for strains B58 and 4607, did not resemble that of other *Brucella* biotypes. This illustrates the point that unless the genetic relationships of micro-organisms can be established by independent means, physiological tests are of little value in identification.

The other properties of these strains further confined their non-identity with brucellas. Strain B58 was found to be smooth, but even so was quite resistant to lysis by the brucella phages studied. These included one phage, S708, which is lytic for *Br. abortus*, *Br. neotomae* and *Br. suis* biotypes 1, 2, 3 and 4 (J. A. Morris & M. J. Corbel; to be published). Strain 4607 was rough and thus not unexpectedly phage-resistant.

Examination of the phenol-soluble proteins of B58 and 4607 by disk electrophoresis according to Razin & Rottem (1967) has shown that they have very similar electrophoretic mobility distributions which are distinct from those typical of the *Brucella* genus (J. A. Morris, to be published).

The serological studies provided the final indisputable evidence for the absence of any relationship of strains B58 and 4607 to the *Brucella* genus. No serological cross-reactivity between brucellas and these strains could be demonstrated by complement fixation, immunofluorescence, immunoadsorption, ring precipitin or immunodiffusion reactions. The apparent cross-agglutination observed with many serum samples was probably due to natural antibodies coincidentally present in the sera. This was confirmed by the frequent occurrence of agglutinins for B58 in sera from 'normal' animals.

The immunodiffusion results clearly established the absence of serological relationship between B58 and 4607 and brucellas. Furthermore the cross-precipitation obtained with polyvalent antisera to *Mima polymorpha* provided confirmatory evidence for the identification of these two strains. Although B58 and 4607 differed from each other in certain cultural and biochemical properties and in antibiotic sensitivity, the reaction of identity obtained with *M. polymorpha* antiserum in immunodiffusion tests confirmed their relationship. *M. polymorpha* comprises a heterogeneous group of Gram-negative non-fermentative organisms most of which have now been reclassified into the *Acinetobacter* and *Moraxella* genera. As both B58 and 4607 were oxidase-positive they should be classified as *M. polymorpha* var. *oxidans* or *Mor. duplex* strains. Their general morphological, cultural and biochemical properties are consistent with this, although B58 is somewhat atypical in view of its resistance to penicillin G. However, antibiotic sensitivity is essentially a mutable characteristic and certainly not a basis for microbial classification. The biochemical activities described for *M. polymorpha* var. *oxidans* show some variation between isolates and between reports by different authors (Schaub & Hauber, 1948; Brodie & Henderson, 1964; Pickett & Manclark, 1965; Gilardi, 1968).

Similarly the lack of pathogenicity of these strains, although at variance with some studies on *M. polymorpha* (Schaub & Hauber, 1948), is consistent with

others (Brooke, 1951) and it seems probable that pathogenicity is a variable characteristic which may be lost on repeated subculture.

The pathological significance of strains B58 and 4607 is uncertain. Their isolation from abortion material is no evidence that they caused the abortions and it can only be stated that the veterinary significance of *M. polymorpha* var. *oxidans* (*Mor. duplex*) is at present uncertain. It is the author's impression that organisms of this group are commonly isolated from pathological material but are usually discarded as contaminants. Clearly further study of their pathogenic significance, particularly in relation to reproductive disorders in animals, is indicated.

In view of the results of the present study, identifying two of the strains representative of '*Br. suis* biotype 5' (Renoux & Philippon, 1969) as *M. polymorpha* var. *oxidans*, it is clear that the taxonomic status of this group should be re-examined.

The two strains B58 and 4607 cannot be considered as examples of a fifth *Br. suis* biotype and this suggests that the proposed group of Renoux & Philippon (1969) is of dubious validity.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Strain B58, showing general morphology. Capsule stain.

Fig. 2. High-power magnification detail of Fig. 1, showing capsules.

Fig. 3. Strain 4607, showing general morphology and similarity to B58. Capsule stain.

Fig. 4. Strain 4607. High-power magnification detail of Fig. 3, showing capsules and chain formation.

PLATE 2

Fig. 1. Electron micrograph of a negatively stained preparation of strain B58. The capsules (*C*) are clearly visible.

Fig. 2. Electron micrograph of strain 4607 prepared as for Fig. 1, showing capsules (*C*). The morphological similarity of strains B58 and 4607 is readily apparent.

PLATE 3

Fig. 1. Immunodiffusion of ultrasonically disrupted extracts of strains B58 (B58 u/s), 4607 (4607 u/s) and *Br. abortus* 544 (544 u/s) against rabbit antiserum to *Br. abortus* 544 (a Br.ab). Extensive reactions were produced by 544 u/s against its homologous serum but no reaction was given by the other strains.

Fig. 2. Immunodiffusion of B58 u/s, 4607 u/s and 544 u/s against antiserum to B58 (a B58). Extensive cross-reactions were apparent between the B58 and 4607 preparations but there was no evidence of any reaction between 544 u/s and this antiserum.

Fig. 3. Immunodiffusion of B58 u/s, 4607 u/s, 544 u/s against rabbit serum a Br.ab and polyvalent antiserum to *Mima polymorpha* (aMP). Reactions of identity were produced between B58 u/s and 4607 u/s on diffusion against aMP, but no reaction was produced on diffusion against a Br.ab. Similarly 544 u/s reacted only with its homologous antiserum and not with aMP.

Fig. 4. Diffusion of B58 u/s, 4607 u/s and ultrasonically disrupted *Moraxella duplex* and *Acinetobacter anitratum* reference strains against serum aMP. A reaction of identity was produced between the B58, 4607 and *Mor. duplex* preparations, but a reaction of non-identity was given between the *Ac. anitratum* preparation and these strains.


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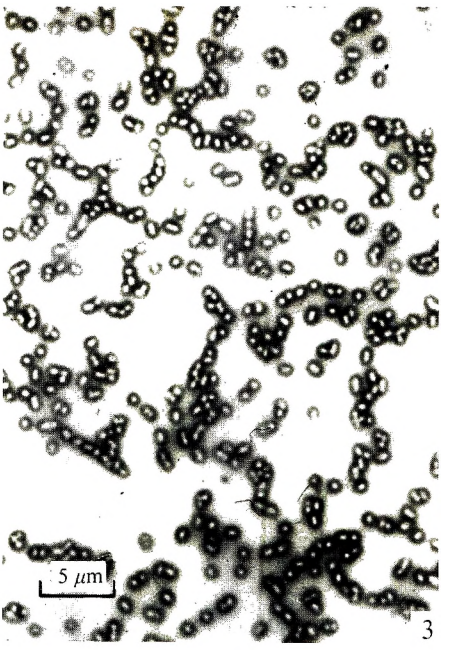
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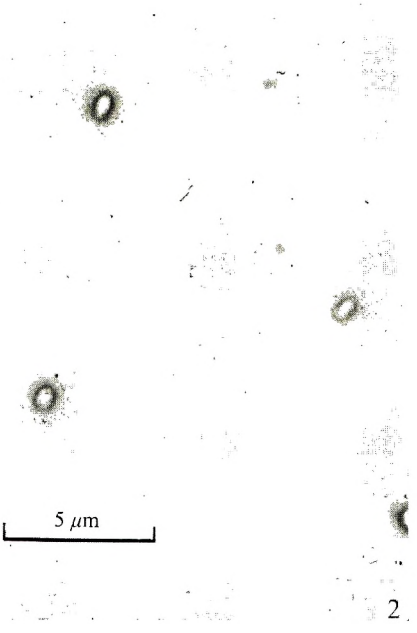
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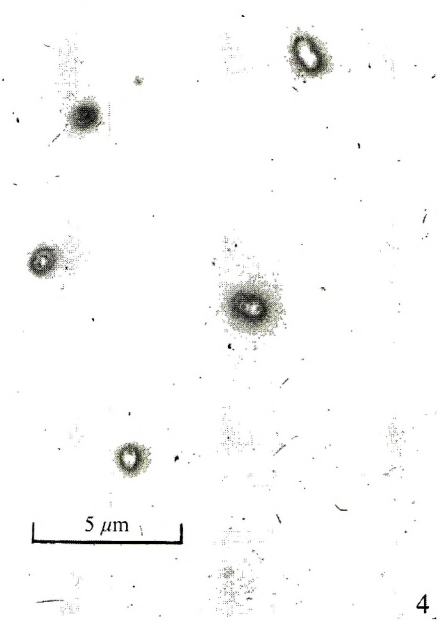
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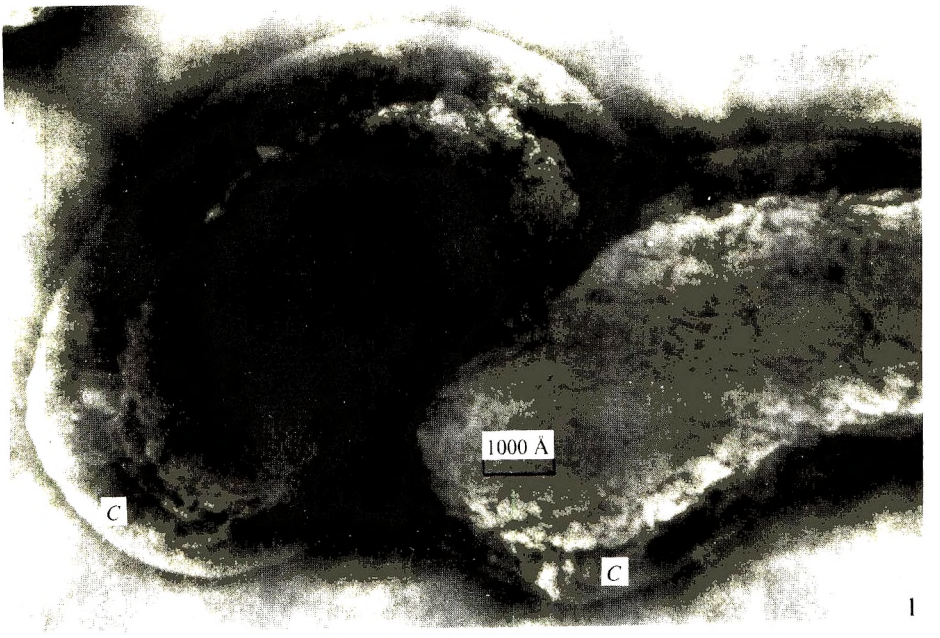

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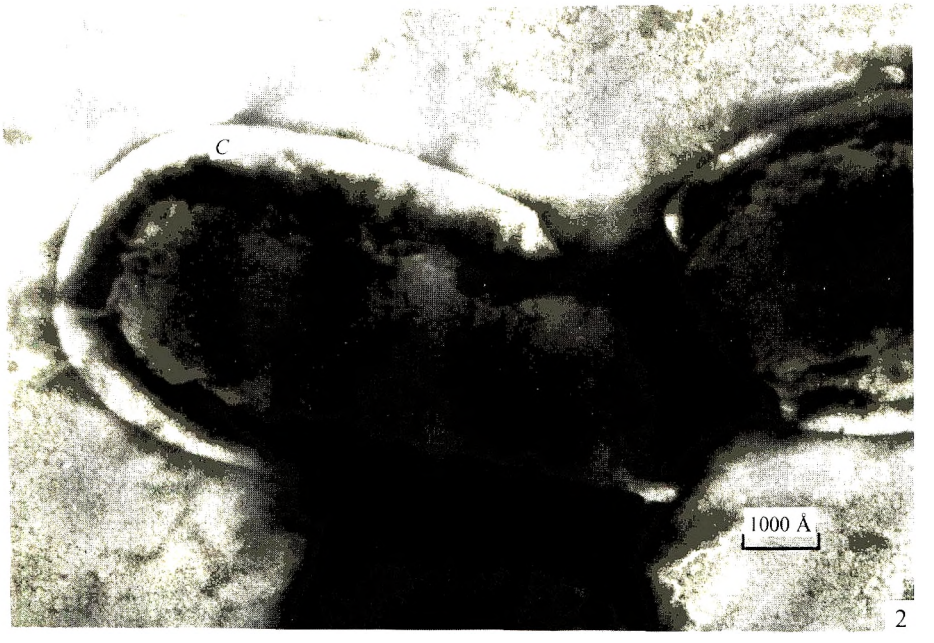

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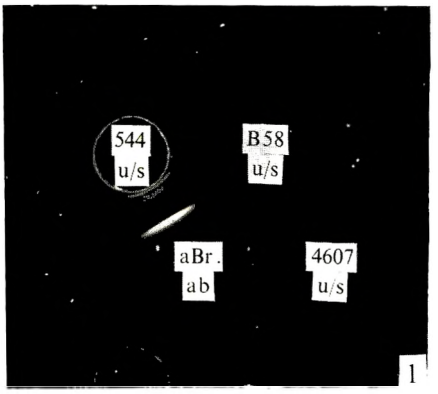
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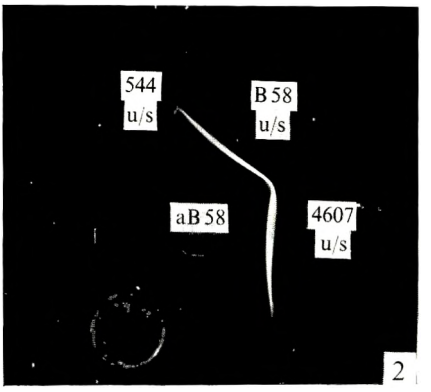
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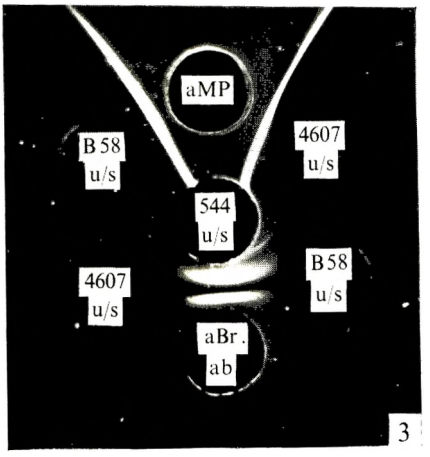
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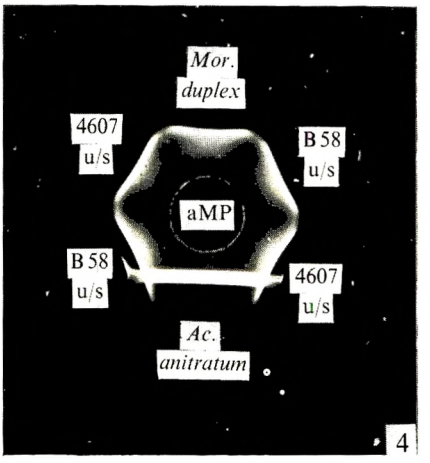
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anitratum
4

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Reactions to tetanus toxoid*

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SUMMARY

In a factory population the occurrence of reactions to tetanus toxoid was recorded after 6740 injections. The incidence of general reactions was 0·3 % and of local reactions 2·6 %. The local reaction rate to the first injection of the basic immunization course was 0·9 %, to the second injection 2·7 %, and to the third injection 7·4 %. To booster injections the rate was 1·6 %. The local reaction rate was appreciably higher in women than in men – 14·4 % and 5·7 % respectively in the case of the third injection – and the incidence among women increased with age.

Tetanus vaccine containing 10 Lf of toxoid caused fewer reactions than one containing 20 Lf, but a reduction in the content of aluminium adjuvant did not affect the reaction rate.

Almost all reactors were found to have a satisfactory serum antitoxin concentration at the time of the reaction or developed a satisfactory immunity within 1–6 months.

Skin tests were made in 32 hypersensitive patients. Neither the diluent, thiomersal preservative, nor the culture medium appeared to be responsible for hypersensitivity. The degree of hypersensitivity elicited by a special highly purified toxoid was only very slightly less than that elicited by the commercially pure toxoid. It is suggested that reactions are largely due to the toxoid antigen itself rather than to impurities or other components of the vaccine.

INTRODUCTION

Although tetanus toxoid is a safe vaccine it has become evident in recent years that its injection is sometimes followed by an adverse reaction (Whittingham, 1940; White & Ungar, 1967; Edsall *et al.* 1967; Fardon, 1967). Whilst severe reactions are rare the development of a painful, swollen area after immunization

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is quite common and does deter people from accepting the vaccine, particularly in a factory or similar environment, where a vaccination procedure can quickly acquire a bad reputation (Griffith, 1967; Yeager, 1967). For this reason we have studied the occurrence of reactions to tetanus toxoid and have attempted to evaluate the role of some of the factors responsible for their development.

MATERIALS AND METHODS

Vaccines and immunization procedures

The tetanus vaccines were aluminium hydroxide-adsorbed preparations (Tet/Vac/PTAH. B.P.) made by the Wellcome Research Laboratories. The three vaccines used contained in each 0·5 ml. dose (1) 0·65 mg. of aluminium ion and 20 Lf of toxoid, (2) 0·65 mg. of aluminium ion and 10 Lf of toxoid, (3) 0·3 mg. of aluminium ion and 10 Lf of toxoid. The different vaccines were given in monthly rotation, each new individual presenting within a given month being started on the same vaccine. Each subject received the same vaccine throughout his course of immunization. An adsorbed toxoid was also prepared containing a specially reduced amount of antigen – 2 Lf toxoid and 0·59 mg. aluminium ion per dose – for use in completion of the course of immunization in reactors.

The subjects, whose ages ranged from 15 to 65 years, all worked at the British Leyland (Austin–Morris) Ltd. factories in Oxford and Abingdon. A history of previous immunization is recorded routinely for all employees, together with details of service in the Armed Forces. It was assumed that such service had led to immunization against tetanus except in those who had served in the British Army between 1945 and 1949 and in the Royal Navy between 1945 and 1958, during which periods tetanus immunization was not officially practised (Ministry of Defence, personal communication). It was also established that since 1960 the injection given at the local hospital at the time of injury has been tetanus toxoid (J. C. Scott, personal communication).

The basic immunization course consisted of three intra-muscular doses of vaccine, spaced with an interval of 6 weeks between the first and second doses and 6 months between the second and third. Booster injections include (*a*) those given at the time of injury to previously immunized patients who had not received a toxoid injection within the previous 12 months, and (*b*) injections given to reinforce immunity in persons who had had the basic course of injections of plain toxoid more than 3 years earlier (White *et al.* 1969). All injections were given by the sisters and nursing staff. A full basic course of immunization was offered to unimmunized employees and also to those immunized more than 20 years previously, those with a vague history of immunization and those with an incomplete history of immunization more than 3 years previously. It should be pointed out that during the period of this study the duration of immunity against tetanus became more clearly defined, and nowadays many workers would agree that those immunized even more than 20 years previously need only have one injection of toxoid in order to boost their waning immunity, and that booster injections at the time of injury are probably unnecessary for perhaps some years after a previous

booster or full course of immunization (Rubbo, 1966; Scheibel, Bentzon, Christensen & Biering, 1966; Adams, Laurence & Smith, 1969; Peebles, Levine, Eldred & Edsall, 1969).

Reactions

The employees were asked to note and report whether any untoward effect occurred after immunization with tetanus toxoid. They were also asked to return to the medical centre 48 hr. after immunization, whether they had a reaction or not, when they were questioned regarding adverse reactions, and the site of injection was examined. We believe that very few patients with reactions were not seen after immunization. Local reactions were examined and the diameters of the areas of induration and of erythema measured using a transparent rule. Reactors were kept under observation until they were free from symptoms, all visible signs had disappeared and no local tenderness was present.

Local reactions were classified in the following way:

(*a*) 'Severe' – when an area of redness and/or oedema greater than 12 cm. in diameter was present at the injection site.

(*b*) 'Moderate' – when an area of redness and/or oedema of between 2 and 12 cm. in diameter was present.

(*c*) 'Negligible' – when an area of less than 2 cm. in diameter was involved. 'Negligible' reactions occurred very rarely; patients either developed a reaction greater in diameter than 2 cm. or developed no visible reaction at all. This category has been ignored in reactions rates quoted in this study.

General reactions were recorded when any untoward symptom was reported, other than those referable to the injection site.

Blood samples

Blood samples were taken for tetanus antitoxin titration:

(*a*) During the basic immunization course from about 1 in 20 of the subjects who were selected randomly. Samples were collected from each of these subjects (1) one week after the first injection, (2) immediately before the second injection, (3) immediately before the third injection and (4) one month later.

(*b*) In patients who had an adverse reaction (1) at the time of the injection or up to 1 week later, (2) one month after the injection or up to 6 months later.

The serum antitoxin concentrations were measured by the method of Glenny & Stevens (1938). In calculating the geometric mean serum antitoxin concentration, less than 0·01 unit/ml. was taken to be 0·007 unit/ml.

Skin tests

Skin tests were made by intradermal injection of approximately 0·01 ml. of the test material – a volume sufficient to give a small clearly visible bleb. Each subject was tested with four injections given at the corners of an imaginary square about $2\frac{1}{2}$ in. wide on the flexor surface of one forearm. The four skin test materials were prepared by the Wellcome Research Laboratories and consisted of:

(1) Vaccine diluent. Sodium borate–succinic acid buffer at ph 6·8, containing thiomersal 1/10,000 as a preservative.

(2) 'Commercial' toxoid. This material is tetanus vaccine in simple solution, B.P. (Wellcome, PX 191C), and is prepared by toxoiding a culture filtrate of *Clostridium tetani*. The toxoid is purified and concentrated and then diluted to 45 Lf/ml. with vaccine diluent. The preparation contained 58 μg. total nitrogen and 33 μg. macromolecular nitrogen per ml.

(3) 'Medium.' Sterile, uninoculated Mueller medium (PX 191B) (Fisek, Mueller & Miller, 1954), processed in a manner identical to the culture filtrate above. It contained 47 μg. total nitrogen and 33 μg. macromolecular nitrogen per ml.

(4) 'Purified' toxoid (PX 191D). This material was prepared by purifying and concentrating the toxin from a *Cl. tetani* culture lysate. The purified toxin was then toxoided and contained 30 μg. total nitrogen and 22 μg. macromolecular nitrogen per ml. when diluted to contain 45 Lf of toxoid per ml.

The skin test sites were observed and the diameters of any resulting areas of induration and of erythema measured with a transparent plastic rule at 30 min. and at 6, 24 and 48 hr. after injection. Skin reactions of less than 3 mm. in diameter were recorded as 0 mm.

RESULTS

General reactions

Only 22 out of 6740 injections (0·3%) were followed by general symptoms. In each case the symptoms developed within 6 hr. of injection and were influenza-like, with headache, lethargy and aching limbs. No general reactions were seen later than 6 hr. after injection and no delayed illness such as serum sickness. No reactions resembling anaphylaxis were seen. In not one instance were we able to decide with certainty that the general reaction genuinely represented hypersensitivity to the toxoid, but they did occur within 6 hr. of the injection, and 10 of the 22 patients also had a local reaction, so that the influenza-like symptoms may well have represented a form of generalized hypersensitivity. One patient had an exacerbation of his asthma within a few hours of a booster injection. One other had a local reaction over the site of his first injection, 6 hr. after his second injection was given into the opposite arm.

Local reactions

Of 6740 injections, 172 (2·6%) were followed by a local reaction greater than 2 cm. in diameter. Of the reactions, 139 were 'moderate', with an area of redness and/or swelling 2–12 cm. in diameter, causing only mild discomfort in the injected arm. Thirty-three patients (0·5%) had 'severe' reactions that measured between 12 and 30 cm. in diameter. The 'severe' reactions were painful and two of the patients had to be put off work for 48 hr. before the swelling and erythema settled under treatment with antihistamine and analgesic. Eight female patients developed a hard, tender swelling at the injection site during the week after immunization – four of these patients were treated with cloxacillin and, whether due to the treatment or not, the swelling subsided in about 7 days.

Table 1. *Incidence of local reactions to adsorbed tetanus toxoid*

	Injections in basic immunization course												Booster injections			
	1st				2nd				3rd							
	No. of injections	No. of reactions	%	S.E.* (%)	No. of injections	No. of reactions	%	S.E. (%)	No. of injections	No. of reactions	%	S.E. (%)	No. of injections	No. of reactions	%	S.E. (%)
Male	1493	6	0·4	0·2	1345	27	2·0	0·4	826	47	5·7	0·8	2025	30	1·5	0·3
Female	379	10	2·6	0·8	343	19	5·5	1·2	195	28	14·4	2·5	134	5	3·7	1·6
Total	1872	16	0·9	0·2	1688	46	2·7	0·4	1021	75	7·4	0·8	2159	35	1·6	0·3

Table 2. *Incidence of local reactions to adsorbed tetanus toxoid by age and sex*

	Men				Women			
Age	No. of injections	No. of reactions	%	S.E.* (%)	No. of injections	No. of reactions	%	S.E. (%)
15–20	320	8	2·5	0·9	205	7	3·4	1·3
21–30	992	23	2·3	0·5	277	15	5·4	1·4
31–40	672	16	2·4	0·6	160	11	6·9	2·0
41–50	813	10	1·2	0·4	166	12	7·2	2·0
51–60	668	16	2·4	0·6	140	11	7·9	2·3
Over 60	331	7	2·1	0·8	6	0	—	—

* S.E. = standard error.

Most reactions developed within 48 hr. of immunization, usually between 12 and 24 hr. Only three reactions appeared between 48 hr. and 7 days of immunization. The reactions remained at the maximum size for about 12–24 hr. and then slowly subsided to disappear in 2 or 3 days. In 11 patients, however, the local reactions showed a progressive further enlargement and were maximal 7–14 days after immunization.

The incidence of local reactions to each of the doses of toxoid is given in Table 1. Only the first occurrence of a reaction is recorded and patients who had a reaction did not normally complete their courses, unless their serum antitoxin proved to be unduly low. The true incidence of reactions to second and third injections is therefore likely to be higher than recorded in Table 1. The incidence of reactions increased with each dose of the basic immunization course and was highest after the third dose (7·4 %), but fell to a lower level (1·6 %) with booster doses.

It may be seen that reactions sometimes occurred after the first injection of tetanus toxoid. Of 16 reactions recorded to the first injection, 6 were beyond reasonable doubt genuinely to the first dose of toxoid, the patients having had less than 0·01 unit of antitoxin/ml. of serum 8–12 days after the injection. Of these 6 reactions, 5 were late, being maximal at 7–14 days after the injection.

The reaction rate in females was found to be more than twice as high as in males. Of the 16 reactors to the first injection 10 were female, and of the 11 patients whose reactions were maximal 7–14 days after the injection, 8 were female.

In Table 1 no distinction is made between individuals who had an incomplete or vague history of previous immunization and those with no history of previous immunization. To discover whether these two categories of subjects had different rates, a random sample of 1000 injections was re-examined and corrected rates calculated for those with some previous immunization and for those with none. The reaction rate was slightly higher among those with no previous immunization histories, but the difference was not significant. Partial immunization some years previously was therefore not found to increase reaction rates in the present study.

Table 2 records the incidence of reactions to the toxoids by age and sex. In women the reaction rate increased steadily with age, whereas in men the rate remained unchanged.

Analysis of the incidence of reactions by season and sex is shown in Table 3. The incidence in females fluctuated much more widely than in males, the highest incidence being nearly 9 % in the April–June quarter, and the lowest being 2·4 % in the July–September quarter. In males the incidence showed no significant seasonal change, being 2·5 % in April–June and 1·4 % in July–September.

A comparison of the incidence of reactions to the three vaccines, containing different amounts of toxoid and adjuvant, is shown in Table 4. When the differences between the reaction rates recorded in Table 4 were tested for significance by the method of Cochran (1954) it was found that vaccine containing 10 Lf caused significantly fewer local reactions than vaccine containing 20 Lf per dose ($P < 0{\cdot}05$). A reduced content of aluminium adjuvant caused no further significant improvement.

The antitoxin response to the three toxoids is recorded in Table 5. It may be

Table 3. *Incidence of reactions to adsorbed tetanus toxoid by season and by sex*

	Men				Women			
	No. of injections	No. of reactions	%	S.E.* (%)	No. of injections	No. of reactions	%	S.E. (%)
Jan.–Mar.	986	22	2·2	0·5	208	13	6·3	1·7
Apr.–June	1695	42	2·5	0·4	297	26	8·8	1·6
July–Sept.	1780	24	1·4	0·3	339	8	2·4	0·8
Oct.–Dec.	1418	22	1·6	0·3	237	16	6·8	1·6

* S.E. = standard error.

Table 4. *Incidence of local reactions to different toxoid preparations*

			Injections in basic immunization course									Booster injections			All injections		
			First			Second			Third								
Toxoid	Aluminium ion content (mg. in dose of 0·5 ml.)	Toxoid content (Lf in dose of 0·5 ml.)	No. of injections	No. of reactions	%	No. of injections	No. of reactions	%	No. of injections	No. of reactions	%	No. of injections	No. of reactions	%	No. of injections	No. of reactions	%
1	0·65	20	111	2	1·8	110	5	4·6	97	4	4·1	125	4	3·2	443	15	3·4
2	0·65	10	453	3	0·7	379	7	1·9	209	5	2·4	674	11	1·6	1715	26	1·5
3	0·3	10	387	4	1·0	310	6	1·9	198	4	2·0	576	11	1·9	1471	25	1·7

Table 5. *Serum antitoxin response to different toxoid preparations*

Toxoid	Aluminium ion content of 0·5 ml. dose (mg.)	Toxoid content of 0·5 ml. dose (Lf)	Relation to basic immunization course: 6 weeks after 1st dose — No. of persons	6 weeks after 1st dose — G.M.T.*	6 months after 2nd dose — No. of persons	6 months after 2nd dose — G.M.T.*	1 month after 3rd dose — No. of persons	1 month after 3rd dose — G.M.T.*
1	0·65	20	21	0.06	17	0.33	20	10.8
2	0·65	10	52	0.03	42	0.16	43	5.3
3	0·3	10	70	0.02	64	0.14	59	4.8

* G.M.T. = geometric mean antitoxin concentration.

Table 6. *Distribution of serum tetanus antitoxin concentration in reactors and matched non-reactors*

Time of antitoxin titration	Subjects	No. of subjects	% with a serum antitoxin concentration (unit/ml.) of: <0·01	0·01–	0·1–	1·0–	10–100	Geom. mean serum antitoxin (unit/ml.)
(A) At or up to 1 week after injection	Male reactors	81	7	11	37	34	11	0·66
	Female reactors	33	30	18	34	15	3	0·10
	Total reactors	114	14	13	36	28	9	0·38
(B) At time of reaction or up to 1 week later	Reactors	68	16	15	35	28	6	0·31
	Matched non-reactors	68	25	22	40	12	1	0·09
(C) At 1 month after reaction or up to 6 months later	Reactors	62	0	5	13	48	34	3·6
	Matched non-reactors	62	2	6	26	39	27	2·2

seen that reduction in the Lf content of the vaccine was accompanied by some diminution in the response to each dose of the basic immunization course. The diminution is significant by the *t* test at the 2% level in the case of the response to the first dose, and at the 5% level in the responses to the second and third doses. A reduction in the content of aluminium adjuvant caused no further significant change in the antitoxin response.

Immune status of reactors

Most of the individuals who had a local reaction to tetanus toxoid were found to have a high serum antitoxin at the time of reaction, but this was not always the case. Table 6A shows that although 73% of reactors had 0·1 unit of antitoxin/ml. of serum or more, 14% had less than 0·01 unit/ml. When reactors were compared with non-reactor controls, carefully matched for age, sex, stage of immunization, vaccine and month in which the vaccine was given (of the 114 reactors whose antitoxin at the time of reaction were known, 68 could be matched), the mean serum antitoxin of reactors was more than three times higher than that of controls (Table 6B).

The possibility was considered that the 14% of reactors who had less than 0·01 unit of antitoxin/ml. of serum might have been in the process of developing a satisfactory antitoxin response. It can be seen in Table 6C that all of 62 matched reactors had more than 0·01 unit/ml. of antitoxin 1–6 months after the injection which had caused a reaction. Antitoxin titrations were also made in another 46 reactors who were not matched, and all but one had over 0·01 unit/ml. of serum 1–6 months after the toxoid injection. The one patient who produced no antitoxin was a female, whose reaction to her first dose of toxoid reached its maximum size ($5 \times 2{\cdot}3$ cm. erythema and 2×1 cm. induration) 13 days after the injection. Four weeks later her serum antitoxin was still less than 0·01 unit/ml.

Some evidence was obtained that the serum antitoxin concentration may rise more rapidly in persons who react to toxoid than in those who do not. When tested 6 weeks after the first injection of toxoid 11 out of 15 reactors (73%) showed a serum antitoxin rise from less than 0·01 unit/ml. to 0·1 unit/ml. or more, whereas only 20 of 104 non-reactors (19%) showed a similar rise. The difference is highly significant ($P = 0{\cdot}00005$). Two reactors showed a rise in their serum antitoxin concentration from 0·01 and 0·02 unit/ml. respectively to 50 units/ml. within a month after injection.

Although the incidence of reactions differed in men and women, and in women of different ages, no similar variation in the antitoxin responses could be detected.

Low dose toxoid

The reduced dose of adsorbed toxoid (2 Lf per dose) has so far been used to complete the course of immunization in 25 reactors. Only 2 of the 25 experienced an adverse response; they were females who had had both a moderate local reaction and generalized symptoms following full-dose toxoid. In these two

patients the low dose preparation caused local reactions with erythema of 7·5 cm. and 2·5 cm. diameter respectively and no generalized symptoms. The serum antitoxin concentrations in the 25 subjects 1 month after the reduced dose were highly satisfactory, having risen from a mean of 0·23 unit/ml. to 5·0 units/ml. with a range of from 1 to 20 units/ml.

Skin tests

Skin tests were made in 32 patients who had developed local reactions of varying severity to an immunizing injection of tetanus toxoid between 3 months and 8 years previously. The two skin-test toxoids were injected in a dilution of 1/20, 1/50, 1/200 or 1/500 according to the severity of the reaction the patient had experienced. One patient, who had had a very small reaction, was tested with undiluted skin-test material, 20 patients with 1/20, 4 with 1/50, 5 with 1/200 and 2 patients, who had had severe reactions to immunization, with 1/500 dilutions.

Two patterns of response to skin-testing were observed:

(*a*) A wheal and flare response which reached a peak at 20–30 min. and then faded slowly and completely – in no patient was any reaction visible at 6 hr.

(*b*) A delayed response consisting of erythema with induration, reaching a peak at about 24 hr.

It is probable that the 30 min. response represents reagin-mediated hypersensitivity, and the 24 hr. response delayed-type hypersensitivity.

In Tables 7 and 8 the results are given in terms of the diameters of the area of erythema at 30 min., and of induration at 24 hr. after injection of the test material; the mean diameters given are calculated from the tests at all dilutions, because there was no significant difference between the means among patients tested with the different strengths of skin-test toxoids.

Skin tests with the thiomersal-containing vaccine diluent and with the 'medium' caused negligible responses in the 32 patients who had previously reacted to an immunizing dose of toxoid. Only two patients responded to the diluent and four to the broth and in each case the responses were small and consisted of a wheal and flare with no delayed component.

All except one of the patients showed a positive response to skin testing with both toxoid preparations. Most of the subjects developed both an immediate and a delayed response, but seven had only an immediate response and four patients only a delayed response. The reactions to the 'purified' toxoid were, in general, slightly smaller than those obtained with the 'commercial' toxoid, and in two patients a small delayed response occurred to the 'commercial' toxoid only.

Skin tests were also made in nine subjects who had been immunized with toxoid but had shown no reaction, and in five unimmunized persons. In these control subjects the skin-test toxoids were injected undiluted (Table 8). Immediate hypersensitivity to the diluent was apparent in one of the non-reactor controls and one of the unimmunized control subjects, but no delayed hypersensitivity was detected. A response to the 'medium' occurred in 6 of the 9 non-reactors and 2 of the 5 previously non-immunized subjects; this reactivity also was only of the immediate type. Delayed hypersensitivity to the toxoid was present in 4 of the 9

Table 7. *Local reactions to skin testing in* 32 *hypersensitive patients*

Skin-test material	Erythema at 30 min.				Induration at 24 hr.			
	Diluent	'Medium'	'Purified' toxoid*	'Commercial' toxoid*	Diluent	'Medium'	'Purified' toxoid*	'Commercial' toxoid*
No. of patients with a reaction to the test material	2	4	26	27	0	0	22	24
Mean diameter of reaction (mm.)	4	4	18	20	0	0	9	11

* The skin test toxoids injected in dilutions of 1/20 (20 patients), 1/50 (4 patients), 1/200 (5 patients) or 1/500 (2 patients), and undiluted in 1 patient, according to the degree of hypersensitivity present in each individual patient.

Table 8. *Local reactions to skin testing in controls: nine persons who had not reacted to toxoid and five previously non-immunized persons*

Skin-test material		Erythema at 30 min.				Induration at 24 hr.			
		Diluent	'Medium'	'Purified' toxoid*	'Commercial' toxoid*	Diluent	'Medium'	'Purified' toxoid*	'Commercial' toxoid*
Nine persons who had not reacted to toxoid	No. of persons with a reaction to the test material	1	6	4	5	0	0	3	4
	Mean diameter of reaction (mm.)	9	9	18	17	0	0	13	10
Five non-immunized persons	No. of persons with a reaction to the test material	1	2	2	2	0	0	0	0
	Mean diameter of reaction (mm.)	9	10	13	16	0	0	0	0

* The skin test toxoids were injected undiluted in all subjects.

immunized persons who had not previously reacted to toxoid injection, but in none of the non-immunized subjects.

DISCUSSION

The reactions encountered in this investigation were those arising during the course of immunizing and maintaining the immunity of a normal factory population. The incidence of reactions was appreciable, and occasional severe reactions, involving the whole upper arm or even the whole arm, prejudiced the success of the campaign.

We still do not know why certain individuals suffer these local reactions or what distinguishes them from non-reactors. There is an increasing predisposition to reactions with increasing immunization, though reactions sometimes occurred to the first injection of toxoid. The reaction rate to boosters was, however, lower than the general reaction rate, which suggests that the state of hypersensitivity revealed when toxoid is given in fairly closely spaced intervals wanes with the passage of time. In our series, known reactors were not given further full doses of toxoid so we have no cases where a reaction to an earlier dose was followed by no reaction to a later full dose. Dr R. G. Orr (personal communication), however, has several such well-documented cases and it is possible that hypersensitivity is sometimes quite short-lived. The high incidence of reactions to the third dose of the basic immunization course might therefore be reduced by adopting a 12-month interval between the second and third doses, rather than a 6-month interval, and we are examining this possibility.

Local reactions to adjuvant vaccines occur less frequently when injections are given intramuscularly rather than subcutaneously (Relihan, 1969; Snell & Burland, 1970), but none of the injections reported here were intentionally given subcutaneously. In this connexion, the greater incidence of reactions among females, and particularly older females, may partly be accounted for by the difficulty of giving them a true intramuscular injection owing to the presence of a deeper layer of subcutaneous fat and smaller muscle mass, although no correlation between depth of fat or muscle mass and reactions was sought in the study reported here. The lower incidence of reactions observed in the summer is unexplained – no other factor could be found to differ in the summer months, such as nursing staff, type of vaccine or effectiveness of follow-up.

The findings presented in Table 4 suggest that the amount of toxoid in tetanus vaccine is an important factor influencing the incidence of local reactions whereas the amount of aluminium adjuvant, within the range examined, was not important. Reduction in the content of toxoid in tetanus vaccine may therefore be a practical way of reducing the reaction rate, although this may be accompanied by some reduction in the antitoxin response (Table 5). Nevertheless, the response induced by the adsorbed vaccine containing 10 Lf of toxoid remained very satisfactory, and such a preparation might therefore be preferred to one containing 20 Lf for routine use.

Patients who react to an injection of toxoid present a problem regarding the

completion of their course of immunization. The examination of the immune status of reactors showed that whilst most had a high serum antitoxin at the time of their reaction, 14% could be regarded as non-immune. Furthermore, whilst the reactors may have a more rapid antitoxin response than non-reactors, the development of circulating antitoxin cannot be regarded as certain, since one of 108 reactors was still unprotected 4 weeks later, although the patient was one of the few who had reacted to the first dose of toxoid. The use of a rapid, easily performed *in vitro* method of antitoxin estimation would be of great value to identify such patients. In the absence of such a technique our findings confirm those of McComb & Levine (1961) and Trinca (1963), that it is possible to avoid further significant reactions and confidently expect the development of a satisfactory antitoxin response in hypersensitive patients by using a small dose of toxoid – for example, 2 Lf. A somewhat different approach has been recommended by Gross, Bartels, Körner & Kindt (1970), who found that the use of plain (non-adsorbed) toxoid for third and booster injections was attended by reduced reaction rates.

A high proportion of those who had had a local reaction to immunization with tetanus toxoid responded to skin testing with toxoid, developing both a wheal-and-flare and a delayed response. A small proportion developed only an immediate reaction or only a delayed reaction to the skin testing, and only 1 of 32 patients had no response. However, a response was not confined to those who had reacted adversely to immunization, because about half the non-reactors tested also responded to the skin tests. It is unlikely therefore that a response to skin testing could be used to forecast which patients might react adversely to vaccination with tetanus toxoid.

The skin-test reaction to the highly purified toxoid was slightly less than that to the commercially purified toxoid, but the difference was small and it seems unlikely that a significant reduction in the reaction rate, or in the severity of local reactions, would follow further purification of tetanus toxoid. A response to skin testing with the thiomersal-containing diluent and to the processed culture medium was uncommon among the reactors and, when it was observed, the response obtained was small and had no delayed component, suggesting that neither is a significant factor in causing reactions to toxoid vaccine. Reactions to thiomersal in skin-test materials are known to occur (Reisman, 1969; Hansson & Möller, 1971*a*), but are not believed to be of importance in determining the development of a reaction to toxoid (Hansson & Möller, 1971*b*). The skin test findings suggest that reactions may largely be caused by the toxoid antigen itself rather than by impurities in the vaccine.

An unexpected observation was the relatively high proportion of the control subjects who showed a reaction to skin-testing with the 'medium'. This reactivity had no delayed component, but it is difficult to explain why a similar response was not seen more frequently in the hypersensitive subjects. It is possible that the reactivity to the toxoids injected about $2\frac{1}{2}$ in. away in the forearm might have impaired a response to the adjacent 'medium'.

In the light of the findings we have discussed, the measures useful in reducing reactions may be summarized as follows:

(1) Accurate recording of immunization histories so that unnecessary courses of immunization and unnecessary booster doses may be avoided.

(2) The use of tetanus vaccine containing 10 Lf of toxoid rather than 20 Lf.

(3) The use of the intramuscular route of injection.

(4) The use of reduced doses of toxoid, e.g. 2 Lf, for those who have previously reacted adversely.

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Macquarie Island: the introduction of the European rabbit flea *Spilopsyllus cuniculi* (Dale) as a possible vector for myxomatosis

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SUMMARY

The European rabbit flea *Spilopsyllus cuniculi* (Dale) was first released on Macquarie Island in December 1968. The flea has survived and bred on the island and about 30 % of the rabbits sampled from the original release area in January 1972 were flea-infested.

INTRODUCTION

Macquarie Island, Fig. 1, situated about half-way between New Zealand and Antarctica (54° 30′ S., 158° 57′ E.), is a long narrow ledge of land about 34 km. long by 4·5 km. wide and 120×10^6 m.^{2} in area. The island rises steeply out of the sea to a plateau ranging in height between 180 and 300 m. with peaks up to 433 m. Weather conditions are bleak, with a mean temperature of 4·4° C. and a mean diurnal range of 3·2° C. The average sunshine of only 1·8 hr. a day is concentrated in the period October–March. The relative humidity seldom falls below 90 % and a figure of 100 % is often maintained for 24 hr. or more. Windy conditions prevail, with an average velocity of about 10 m. s.$^{-1}$ and gusts exceeding 27 m. s.$^{-1}$ frequent at all times of the year. Although devoid of trees the island has a luxuriant vegetation including flowering grasses and broad-leafed succulents.

Rabbits, *Oryctolagus cuniculus* L., were introduced onto many of the sub-Antarctic islands during the latter half of the nineteenth century by sealers and whalers as a source of fresh meat more acceptable than penguin, seal or sea-elephant tongue. The first rabbits released on Macquarie Island were dispatched by William Elder of the Otago Whaling Company in 1879 or 1880 and were described as 'French rabbits' (Cumpston, 1968). They were of domestic origin (Shipp, Keith, Hughes & Myers, 1963). Fluctuating rabbit density was reported and often attributed to predation by feral cats. Another reason for the population

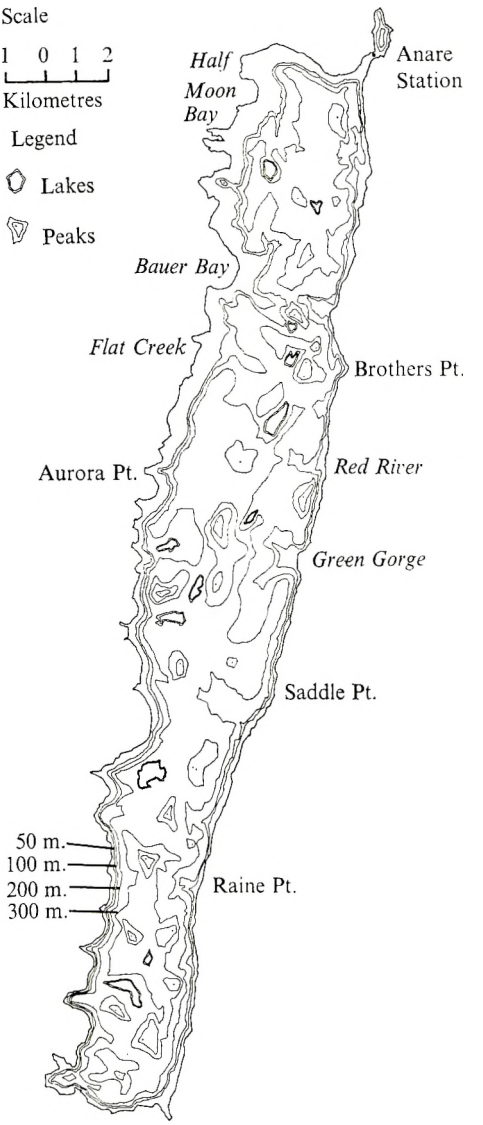

Fig. 1. Illustrating how Macquarie Island rises steeply from sea level to a plateau ranging between 180 and 300 m. in height.

fluctuations could have been pasture over-grazing by rabbits, resulting in denuded areas and consequent movements of rabbits to less denuded areas as suggested by Taylor (1955). Further, the amount of breeding in the denuded areas was found by Shipp *et al.* (1963) to be considerably reduced. Over-grazing by rabbits of the principal dominant, *Poa foliosa*, the only native species capable of stabilizing the soil on steep slopes, results in soil erosion, particularly in the form of soil slips in high moor peat areas. Rabbit over-grazing is also considered to be endangering the survival of a number of plant species (Costin & Moore, 1960; Holdgate & Wace, 1961; Taylor, 1955).

No systematic attempt has yet been made to reduce rabbit numbers on the island, although investigations into possible methods of control have been made (G. C. Johnston, unpublished data). The Glenfield strain of myxoma virus was introduced into about 100 burrows in aerosol form and 15 rabbits were injected with the virus. Reduced burrow activity suggested a localized kill but no sick rabbits were seen. Consistent with this observation, an examination of ectoparasites on rabbits at the time revealed one, *Haemodipsus ventricosus* (G. C. Johnston, 1965, unpublished data), as a possible vector for myxomatosis (Mykytowycz, 1958) at the level of contact infection. Thus in the absence of a suitable mobile vector, myxomatosis could not be considered as a useful means of rabbit control on the island. Poisoning with sodium fluoroacetate ('1080'), in the form of 'one-shot' oat bait (Gooding & Harrison 1964), proved moderately successful in terms of rabbits killed but presented logistic problems as the bait had to be carried and distributed by the operator over very difficult terrain. There was no evidence of poisoning of any natural fauna, although the carcases of two cats were found after poisoning. Burrow fumigation presented difficulties with regard to fauna conservation since rabbit burrows and bird-nest openings were often indistinguishable and co-habitation by rabbits and birds was observed (Johnston, 1966).

Clearly, a biological control which would limit rabbit numbers would be desirable as conventional rabbit control measures are difficult to use on Macquarie Island. The island is devoid of mobile biting insects (Watson, 1967), possibly because it is a narrow, wind-swept island and without such insects the usefulness of myxomatosis as a control measure seemed very doubtful. It therefore seemed a good idea to examine the possibility of introducing the European rabbit flea, *Spilopsyllus cuniculi* (Dale), as a vector for myxomatosis which, if successful, might provide a possible biological control of the rabbit.

Collecting data from a remote island of rugged terrain and unpredictable climatic conditions is difficult and often hazardous. Lone expeditions on the island are forbidden. A supply ship visits the island twice a year, in March and November, and periods ashore are limited to 3–4 days during turn-around (if the weather is fine), 4 months November–March, or 12 months November–November. Long-term observations by an individual are difficult to arrange. The data presented in this paper were collected by the different authors at different times with the help of numerous unnamed members of the Australian National Antarctic Research Expedition (A.N.A.R.E.) stationed on the island with a permanent base on the northern peninsular (see Fig. 1).

MATERIALS AND METHODS

Fleas

All the fleas taken to Macquarie Island were laboratory-bred fleas (Sobey & Conolly, 1971). In transit, fleas were refrigerated at 4° C. They were disseminated within 3 days of their arrival on the island. During 1971/2 6600 fleas collected from rabbits shot on the island were re-released. A small battery-operated aspirator was used to collect fleas off combs.

Blood samples

Blood samples from shot rabbits were collected by the method described by Sobey, Conolly and Adams (1966).

Rabbit ages

Eye lenses were collected for age determination (Lord, 1959; Myers & Gilbert, 1968).

RESULTS

Rabbit distribution

Rabbit distribution for the years 1956, 1965–6 and 1970–1 are shown in Fig. 2. Rabbits were distributed over the whole length of the island throughout the period of observations, but the areas of high density changed with time.

Estimates given by individual A.N.A.R.E. personnel of the numbers of rabbits in the areas labelled 'abundant' varied from 20 to 400 per 400 m.^{2}. Daylight counts made in April 1972 of rabbits seen in an area of approximately $1{\cdot}67 \times 10^4$ m.^{2} at each of the locations Half Moon Bay, Flat Creek, Red River and Green Gorge (see Fig. 1) are shown in Table 1. These are areas classified as 'abundant'. The counts are repeatable. In order to arrive at some estimate of the number of rabbits on the island the areas of the different density subdivisions have been estimated from Fig. 2 and these are presented in Table 2. The highest density was observed in 1965–6 and the lowest in 1956. The area of the island is about 120×10^6 m.^{2}. If the 'abundant' density is taken as 20 rabbits per 400 m.^{2} and the 'common' as 5 rabbits per 400 m.^{2}, estimates of about 50,000 rabbits in 1956 and 150,000 rabbits in 1965–6 for the whole island may be assumed.

Predators

There are three predators of rabbits on Macquarie Island: the feral cats (*Felis catus* L.), the Stewart Island Weka (*Gallirallus australia scotti*) and the Southern Skua (*Catharacta skua lonnbergi*). Cats were reported on the island in 1820, 60 years before rabbits were introduced (Cumpston, 1968). While it is clear that they can survive on the island in the absence of rabbits they are nevertheless considered to be an important predator of rabbits (Fenner & Ratcliffe, 1965).

Wekas, a flightless rail species, were introduced to the island in 1874 (Cumpston, 1968) from Stewart Island, New Zealand. Their distribution largely coincides with the distribution of *Poa foliosa*, and thus they are largely restricted to the coastal fringe (Fig. 3). They feed particularly on earthworms, kelp-fly maggots and small intertidal isopods. Wekas are opportunist feeders and are able to kill young rabbits.

In Plate 1A an adult weka is seen with a 542 g. rabbit kitten it has just killed.

The indigenous skuas are versatile and opportunistic in their choice of animal foods and quick to feed on a shot rabbit. Skuas were often observed to chase rabbits and they prey on both adults and small sickly animals. Rabbit remains were found in many skua-nest territories (Simpson, 1965). Plate 1B shows a skua feeding on an adult rabbit.

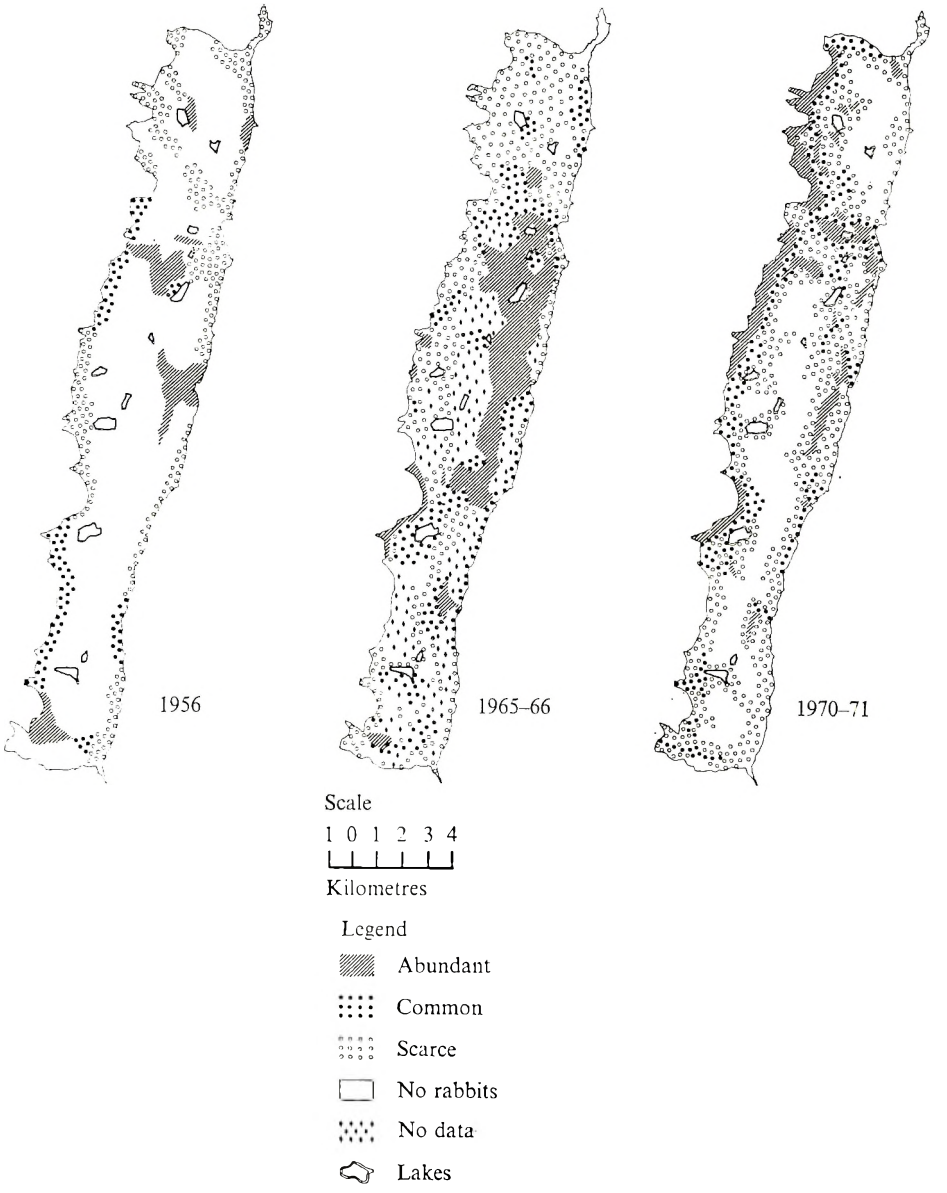

Fig. 2. The estimated density distributions of rabbits in the years 1956, 1965–6 and 1970–1.

Flea releases and recoveries

The first releases of fleas on the island were made in December 1968. The areas where these and subsequent releases were made are shown in Fig. 4A. During the period December 1971 to March 1972, 6600 fleas were collected from shot rabbits and re-released in the north of the island. Between 20 and 200 fleas were collected from females and 10–30 from males, although occasionally 200 were collected from a male. The areas where flea-infested rabbits were recovered are shown in Fig. 4B and the numbers of fleas released and flea-infested rabbits recovered are

Table 1. *Rabbit counts made in April* 1972 *by A.N.A.R.E. personnel in 'abundant' rabbit areas in different locations based on an estimated* $1{\cdot}67 \times 10^4$ m.^{2} *in each case*

Location counted	Count ($1{\cdot}67 \times 10^4$ m.^{2}, 4 acres)
Half Moon Bay	77
Flat Creek	74
Red River	64
Green Gorge	84

Table 2. *The areas of each rabbit density subdivision estimated from Fig.* 1 *and expressed as a percentage of the area of the island*

	Area as a percentage of the island		
Rabbit density	1956	1965–6	1970–1
Abundant	8	18	13
Common	8	19	15
Scarce	23	38	44
No rabbits	61	8	28
No data	—	17	—

shown in Table 3. Clearly the fleas have survived and bred on the island and the numbers of flea-infested rabbits have increased since the fleas were first released.

Rabbit ecology

Very little is known about the ecology of the rabbit on Macquarie Island. Kittens and young rabbits are very rarely seen on the island and this observation may be related to predation. The age structure of the rabbits on the island has not been determined in detail because the rabbits are of domestic origin and lens weights follow a different age/weight curve from that determined for wild rabbits in Australia (Myers & Gilbert, 1968). Only ten out of a sample of 444 lenses collected in January 1972 and aged on an age/weight curve determined from laboratory rabbits (W. R. Sobey, unpublished data) were less than 1 year old.

A very interesting problem of age distribution was found in a separate investigation by G. C. Johnston. He discovered (unpublished data) that lens weights of rabbits on Macquarie Island greatly exceeded those found for any wild rabbits and suggested that many of the rabbits could be old, possibly 5 years or more. In the present samples lens weights exceeding any previously found for laboratory rabbits up to 3 years of age were discovered.

During 1956, while digging out burrows in order to capture rabbits, it was observed that a number of burrows contained nests in which the kittens had been drowned.

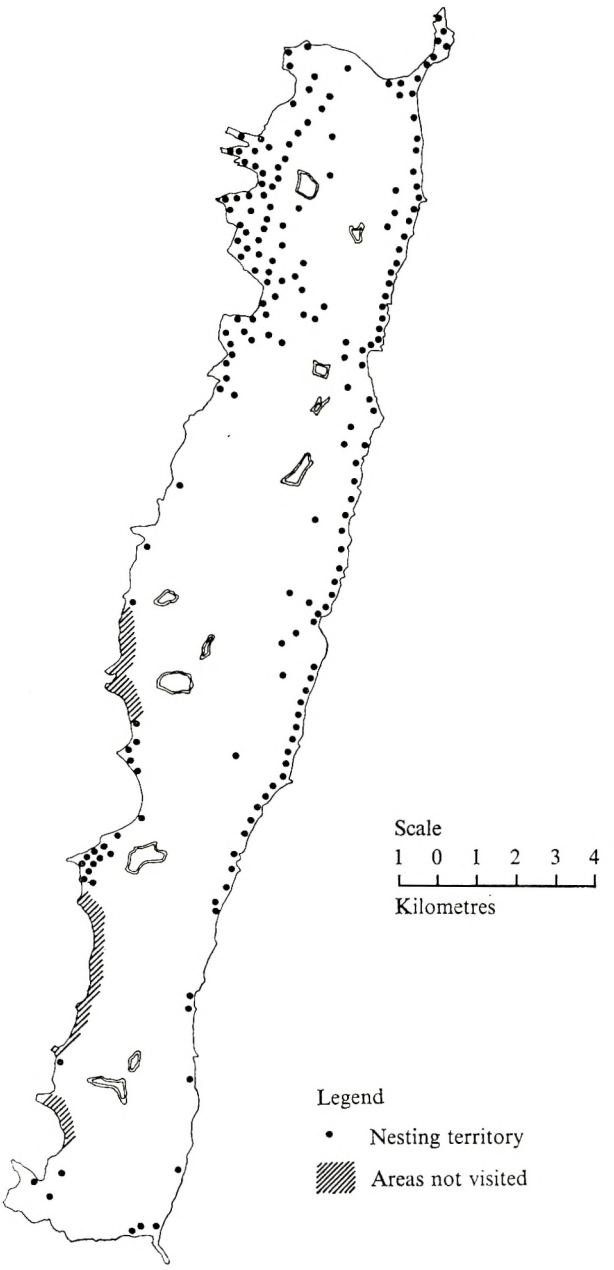

Fig. 3. The distributions of the Stewart Island weka, 1964–6.

Blood samples

A total of 640 blood samples were examined for the presence of antibodies to the soluble antigens of Myxoma virus and all were negative. An examination of the immunoglobulin allotypes whose frequency suggests a founder effect are reported elsewhere by Curtain, Wood & Sobey (1972).

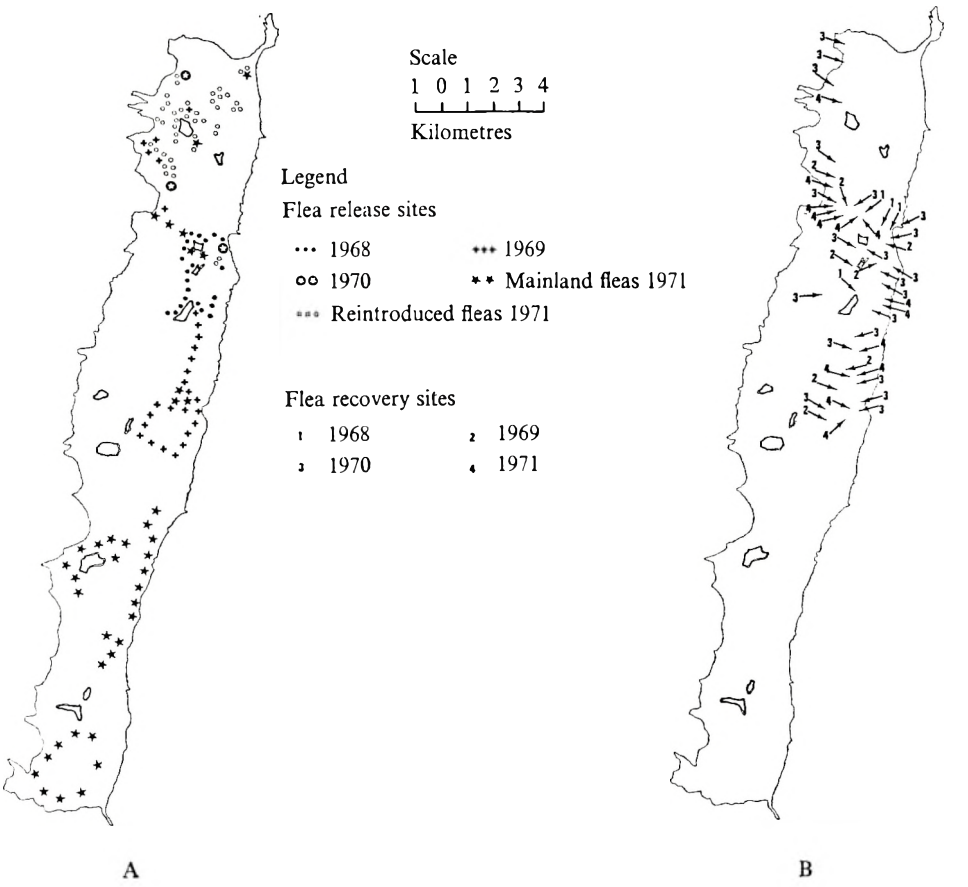

Fig. 4. (A) Flea release sites during the years 1968–72. Fleas were released directly into burrows in lots of 100 or 200. (B) Flea recovery sites during the years 1968–72. The southern half of the island was not sampled in 1971 after fleas were released.

Table 3. *Fleas released on Macquarie Island between* 1968 *and* 1972 *together with the proportion of rabbits in shot samples which were flea infested*

(In 1971, 6600 of the 16,600 fleas released were collected from rabbits on the island and re-released.)

Year	Fleas introduced	Rabbits with fleas	Sample size	Flea-infested rabbits (%)
1968	10,000	—	—	—
1969	10,000	14	624	2
1970	6,000	50	1418	3·5
1971	16,600	386	1879	21
1972	—	109	356	31

DISCUSSION

The high proportion of adults in the population and their extended age-range as compared to that of the Australian mainland rabbits suggests either heavy predation of young with an absence of predation of adults or some high disease or environmental mortality of young rabbits associated with the inhospitable environment. The presence of known predators in the form of cats, wekas and skuas makes predation a likely explanation, at least in part. The high water table, with resulting boggy nature of the ground and the observation that kittens are drowned, offers another direct explanation.

The large fluctuations in population density reported here and in the literature could be related to predation or the effects of selective over-grazing or both.

The extended ages of the adult rabbits suggests a low rate of predation of adults and therefore fluctuations in population density are most likely due to the eating-out of areas by rabbits with movement and decrease of the population. Subsequent regeneration of the vegetation occurs, but there is a continual deterioration of the vegetation as a result of soil erosion and fears have been expressed for the survival of some plant species.

The European rabbit flea was introduced onto the island in 1968 as a possible vector for myxomatosis. Initial recoveries of flea-infested rabbits in 1969 and 1970 were disappointing but by 1972 30 % of the rabbits sampled in release areas were infested. There seems little doubt that the flea has survived and bred on the island. When all, or nearly all, of the rabbits on the island are flea-infested it is proposed to introduce myxoma virus. Being a fully susceptible population a high mortality should result, although it is unlikely that all rabbits will be killed even if all infected rabbits die. Regeneration of the rabbit population should be slow, particularly if predation of young is important. None of the three predators is rabbit-dependent for its food and is not, therefore, likely to diminish in numbers; but the relative predation pressure should be great, resulting in a reduced build-up of rabbit numbers. If the virus remains active as a result of reactivation (Williams, Dunsmore & Parer, 1972) or by retention on the mouthparts of fleas (Chapple & Lewis, 1965) it could act as a continuing biological control, decreasing slowly in effectiveness as genetic resistance (Sobey, 1969) builds up. If, however, the virus disappears after the first outbreak it could be effectively reintroduced when the regenerating rabbit population reached a level sufficient to warrant further control.

We wish to thank the Director, Antarctic Division, Department of Supply, Australia, for the facilities afforded to make this project possible and to the members of the numerous expeditions who have assisted either by personal participation or by providing information from their observations.

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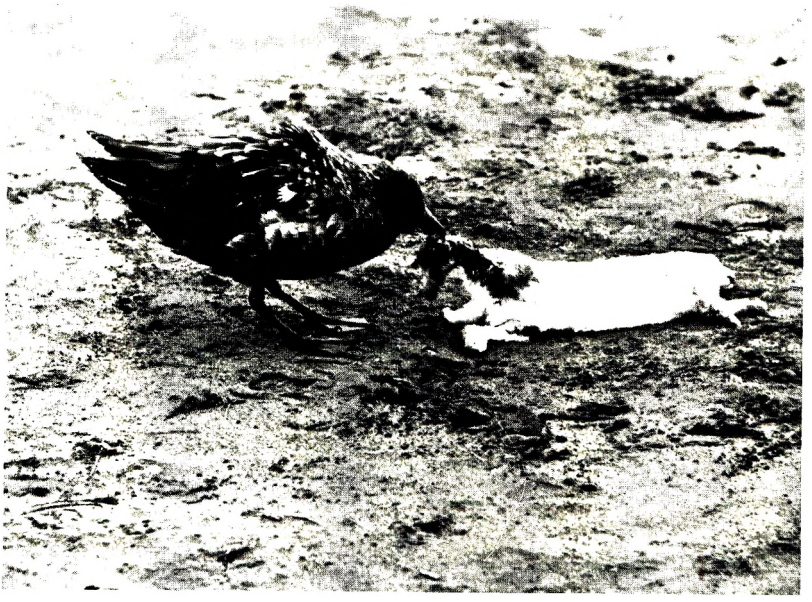
EXPLANATION OF PLATE

(A) An adult female weka with a 542 g. rabbit kitten it has just killed.

(B) A skua feeding on an adult rabbit.

Anare photo: K. N. G. Simpson



Anare photo: K. N. G. Simpson

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The nature of the antibody response to *Yersinia enterocolitica* serotype IX in cattle

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SUMMARY

The nature of the antibody response of cattle to the antigen of *Yersinia enterocolitica* IX cross-reacting with *Brucella* spp. was examined. Density-gradient ultracentrifugation, ion-exchange chromatography, antibody adsorption and elution and disulphide bond reduction tests showed that both 19 S IgM and 7 S IgG_1 and IgG_2 antibodies were produced in response to the cross-reacting antigen. The highest titres of cross-reacting antibodies were detected by the agglutination and Coombs antiglobulin tests. Production of complement-fixing and precipitating antibodies cross-reacting with *Br. abortus* was transient and high titres were not attained.

In contrast, although infection with *Br. abortus* also evoked cross-reacting antibodies of the IgM and IgG classes, much higher titres were produced in the complement fixation and precipitation tests and these persisted for long periods. At all stages of the serological response to both organisms, the two infections could be differentiated by the quantitative Rose Bengal plate test.

INTRODUCTION

The serological cross-reaction between *Yersinia enterocolitica* serotype IX and *Brucella abortus* was first described by Ahvonen and colleagues (Ahvonen, Jansson & Aho, 1969; Ahvonen & Sievers, 1969). Subsequently this cross-reaction was shown to involve other smooth *Brucella* strains (Corbel & Cullen, 1970; Hurvell, Ahvonen & Thal, 1971). Corbel & Cullen (1970) also showed that antibodies evoked by *Br. abortus* could be distinguished from those evoked by *Y. enterocolitica* IX on the basis of a rapid quantitative slide agglutination test using standardized suspensions of Rose Bengal stained *Br. abortus* and *Y. enterocolitica* IX as antigens.

Although this information is of value in cases where infection with *Y. enterocolitica* IX is suspected, it is still important to know the extent to which cross-reacting antibodies evoked by this organism would be likely to interfere with the diagnosis of bovine brucellosis by the standard system of testing employed for the Brucellosis Incentives and Eradication Schemes of the Ministry of Agriculture, Fisheries and Food.

Fundamental to this problem is the nature of the antibody response to the antigen complex of *Y. enterocolitica* IX which cross-reacts with *Brucella* spp. agglutinogens.

Diaz, Jones, Leong & Wilson (1968) considered the agglutinogen complex of smooth brucellas to be equivalent to the phenol-soluble lipopolysaccharide-protein complex of the organisms. Subsequently Diaz, Lacalle, Medrano & Leong (1970) reported that a phenol-soluble endotoxin component from *Y. enterocolitica* IX cross-reacted serologically with *Brucella* spp. agglutinogens. More recently it has been found that the cross-reacting antigen complex of *Y. enterocolitica* IX contains two components reacting with antisera to *Br. abortus* (M. J. Corbel, to be published). Only one of these appears to be strongly agglutinogenic, however, and this is similar in properties to the lipopolysaccharide described by Diaz *et al.* (1970).

The nature of the antibody response evoked by bacterial lipopolysaccharides varies unpredictably between strains and to some extent with the species of animal used for inoculation. Thus in man, *Salmonella typhi* endotoxin only evokes IgM antibodies even on repeated injection (LoSpalluto, Miller, Dorward & Fink, 1962). Similar observations have been made on other *Salmonella* and *Proteus* strains, although the lipopolysaccharides of some evoke both IgM and IgG antibodies (Bauer & Stavitsky, 1961; Pike & Schulze, 1964; Jonas, 1969; Smith, Barnett & Sanford, 1970).

The serological diagnosis of bovine brucellosis is largely based upon observations that infection is associated with complement-fixing IgG antibodies, whereas residual vaccinal antibodies are of agglutinating, but non-complement-fixing IgM type (Anderson, Jenness, Brumfield & Gough, 1964; Schimmel & Erler, 1967). Thus the nature of the cross-reacting antibodies evoked by *Y. enterocolitica* IX is obviously of potential significance in relation to the diagnosis of bovine brucellosis. The object of the present study was to characterize the antibodies produced by cattle in response to experimental inoculation with *Y. enterocolitica* IX.

MATERIALS AND METHODS

Antigens

The bacterial strains and agglutinating antigens used were as described by Corbel & Cullen (1970) except that the *Y. enterocolitica* IX suspensions were standardized to give 50% agglutination with a 1/500 dilution of the International Standard *Brucella abortus* antiserum. Lipopolysaccharide was extracted from *Y. enterocolitica* IX organisms using 88% (w/v) phenol essentially according to Westphal, Lüderitz & Bister (1952). After prolonged dialysis against distilled water, the soluble material recovered from the phenol phase was chromatographed on Sephadex 4B (Pharmacia, Uppsala) according to Romanowska (1970). Fractions appearing immediately after the void volume were concentrated by drying from the frozen state. Lipopolysaccharide was extracted from *Br. abortus* strain 99 according to Leong *et al.* (1970) and purified as for *Y. enterocolitica* IX lipopolysaccharide.

Antisera

Bovine antisera to *Y. enterocolitica* IX were obtained from cattle inoculated as described by Corbel & Cullen (1970). The anamnestic response to this organism

was examined by injection of a second dose of *ca.* 10^{11} organisms by the subcutaneous route *ca.* 12 weeks after the primary injection. Blood samples were collected at frequent intervals thereafter.

Antisera to *Br. abortus* strain 19 and to virulent field strains of *Br. abortus* biotype 1 were obtained as described previously (Corbel & Cullen, 1970). In addition, bovine antisera to the *Br. abortus* strain 544 were prepared by subcutaneous injection of *ca.* 10^{11} viable organisms followed by collection of blood samples at daily intervals for the first 10 days and twice-weekly intervals thereafter.

Serological methods

The serum agglutination (SA) and complement fixation (CF) tests were performed according to standard procedures (Morgan *et al.* 1971). The Coombs antiglobulin and 2-mercaptoethanol tests were performed as described by Morgan (1967). Immunodiffusion tests against *Y. enterocolitica* IX and *Br. abortus* lipopolysaccharides and ultrasonic extracts were done according to Corbel & Cullen (1970) but using 1% (w/v) Oxoid No. 1 agar in 1·0 M-NaCl as diffusion medium. Quantitative Rose Bengal plate (QRBP) tests using standard *Y. enterocolitica* IX and *Br. abortus* antigens were also done according to Corbel & Cullen (1970).

Serum fractionation

Density-gradient ultracentrifugation was performed essentially according to Cowan & Trautman (1965). Fractions were dialysed against phosphate-buffered saline (PBS; 0·15 M-NaCl, buffered at pH 7·2 with 0·01 M phosphate buffer) before testing. Ion-exchange chromatography on DEAE-cellulose was done essentially according to Porter & Noakes (1970).

Immuno-adsorption and elution of antibodies was achieved by absorption of 10 ml. volumes of serum with 1·0 ml. volumes of *Y. enterocolitica* IX or *Br. abortus* S99 suspensions (*ca.* 10^{12} organisms/ml.) at 37° C. for 2 hr. followed by overnight incubation at 4° C. After centrifugation at 15,000*g* for 5 min. the deposited organisms were washed by 3 cycles of centrifugation in PBS. Immunoglobulins were eluted by resuspension of the washed organisms in 0·15 M-NaCl buffered at pH 1·0 with formic acid-HCl buffer (Corbel, 1972).

Analytical methods

Protein was estimated according to Sutherland, Cori, Haynes & Olsen (1949) using crystallized bovine serum albumin (Sigma, London) as standard.

Disk electrophoresis was done according to Davis (1964). Immunoelectrophoresis was performed according to Scheidegger (1955).

RESULTS

The serological response to the cross-reacting antigen of Y. enterocolitica *IX*

As shown in Table 1, injection of cattle with *Y. enterocolitica* IX induced, after 6 days, formation of agglutinins reacting with the homologous organisms and with

Table 1. *The serological response of cattle inoculated with* Y. enterocolitica *IX to* Y. enterocolitica *IX and* Br. abortus *antigens*

		Reciprocal titres in tests with *Br. abortus* antigen							Reciprocal titres in tests with *Y. enterocolitica* IX antigen						
							Precipitins							Precipitins	
Day	Sample	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps
0	Y 1	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	Y 2	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	Y 3	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	Y 4	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
6	Y 1	40	40	< 10	< 2	< 1	0	0	80	80	10	< 2	4	0	0
	Y 2	40	40	< 10	< 2	< 1	0	0	80	80	< 10	< 2	< 1	0	0
	Y 3	160	160	10	20	8	0	0	1280	1280	80	20	16	0	0
	Y 4	160	160	10	20	1	0	0	160	160	< 10	10	8	0	0
10	Y 1	40	40	< 10	< 2	< 1	0	0	80	80	20	< 2	1	0	0
	Y 2	40	40	< 10	< 2	< 1	0	0	80	80	10	< 2	2	0	0
	Y 3	160	320	20	10	8	0	1	640	2560	80	10	64	0	1
	Y 4	320	320	20	10	8	0	0	640	1280	40	10	64	0	1
14	Y 1	40	40	10	< 2	2	0	0	80	80	40	< 2	4	> 1	0
	Y 2	40	40	20	< 2	< 1	0	0	80	80	40	2	2	1	0
	Y 3	160	320	40	20	8	0	1	640	2560	160	20	256	> 2	1
	Y 4	320	320	40	10	8	0	0	640	2560	160	20	512	> 2	1
28	Y 1	40	80	10	< 2	1	0	0	80	80	10	< 2	1	> 1	0
	Y 2	20	40	10	< 2	< 1	0	0	40	40	10	< 2	< 1	> 1	0
	Y 3	80	160	10	< 2	1	0	0	320	640	40	2	4	> 2	1
	Y 4	40	160	10	< 2	1	0	0	80	160	80	< 2	4	> 2	1
49	Y 1	20	40	10	< 2	1	0	0	40	40	< 10	< 2	1	> 1	0
	Y 2	20	20	10	< 2	< 1	0	0	40	40	10	< 2	2	1	0
	Y 3	40	160	10	10	1	0	0	80	80	10	< 2	2	> 2	0
	Y 4	80	160	10	< 2	1	0	0	80	80	< 10	< 2	2	> 1	0

ssa = subsurface antigens. lps = lipopolysaccharide antigen.

* 2-Mercaptoethanol reduction test.

Br. abortus. These agglutinins were largely destroyed by reduction with 2-mercaptoethanol. Antibodies active in the Coombs antiglobulin, RBP, CF and immunodiffusion tests were also detectable at this stage in two of the four sera tested.

On the tenth day after inoculation, the agglutinin titres to both organisms had increased but the agglutinating activity was only partially susceptible to reduction. Antibodies active in the Coombs antiglobulin test and RBP test were detectable to low titre but CF and precipitating activities were absent. The titres in all tests continued to increase until the fourteenth day when they reached their maximum. At this stage precipitating antibodies to both *Y. enterocolitica* IX and *Br. abortus* were detectable by the immunodiffusion test. After this time the antibody titres in all tests steadily declined but the CF and precipitin titres declined more rapidly than the agglutinating antibodies. At this stage and until the titres were finally unmeasurable, the agglutinating activity was partly stable to 2-mercaptoethanol reduction. The agglutination reactions declined to insignificant titres within 2 months of inoculation. The antibodies which persisted for longest were those detectable by the antiglobulin test. However, this may have merely reflected the relatively high sensitivity of this test.

The serological response to the cross-reacting antigen of Br. abortus

The response to infection with both virulent and attenuated strains of *Br. abortus* was very different from that to *Y. enterocolitica* IX with respect to the chronological development of titres in the various tests and in the persistence of these reactions.

As shown in Table 2, cattle inoculated with *Br. abortus* strains 19 or 544 responded by producing agglutinins within 6 days. These were labile to 2-mercaptoethanol but by the tenth day most of the agglutinating activity was reduction-stable. Antibodies active in the CF and antiglobulin tests were detectable by the sixth day, increasing in titre until reaching a maximum at the end of the fourth week after inoculation with *Br. abortus* strain 19. Thereafter the titres declined slowly over a period of 3 months but were still significantly raised at the end of 6 months. The antibodies active in the CF and antiglobulin tests produced in response to inoculation with *Br. abortus* strain 544 showed a similar trend but did not reach a maximum until 6 weeks after inoculation. They underwent only a marginal decline over the succeeding 6 months.

Precipitins became detectable during the second week after inoculation with either *Br. abortus* strain 19 or 544, those reacting with the lipopolysaccharide antigens of *Y. enterocolitica* IX and *Br. abortus* becoming detectable before antibodies to the intracellular antigens of *Br. abortus*. Precipitins to the intracellular antigens of *Y. enterocolitica* IX did not develop. The precipitin patterns to *Br. abortus* antigens increased in complexity over the succeeding 2–3 months and remained apparently unchanged thereafter in animals inoculated with *Br. abortus* strain 544. Sera from cattle vaccinated with *Br. abortus* strain 19 did not possess precipitins when examined 2 years after vaccination.

Comparison of the results of the QRBP test on sera from cattle inoculated with *Br. abortus* with those for sera from *Y. enterocolitica* IX-inoculated animals

Table 2. *The serological response of cattle inoculated with* Br. abortus *strain* 19 *or* 544 *to* Y. enterocolitica *IX and* Br. abortus *antigens*

		Reciprocal titres in tests with *Br. abortus* antigen							Reciprocal titres in tests with *Y. enterocolitica* IX antigen						
							Precipitins							Precipitins	
Day	Sample	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps
0	B 1†	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	B 2†	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	B 3‡	10	< 10	< 10	< 2	< 1	0	0	10	< 10	< 10	< 2	< 1	0	0
	B 4‡	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
6	B 1	80	160	< 10	4	2	0	0	80	80	< 10	< 2	2	0	0
	B 2	160	160	10	10	4	0	0	160	160	< 10	< 2	2	0	0
	B 3	80	160	< 10	4	2	0	0	80	80	< 10	< 2	2	0	0
	B 4	80	80	< 10	10	4	0	0	80	80	< 10	< 2	2	0	0
10	B 1	320	1280	160	40	8	0	1	320	640	40	2	4	0	1
	B 2	320	1280	320	200	16	0	1	320	640	80	40	16	0	1
	B 3	160	320	20	10	8	0	1	160	160	< 10	2	4	0	1
	B 4	80	160	< 10	10	4	0	1	80	80	< 10	2	4	0	0
14	B 1	640	2560	320	200	16	0	1	640	2560	160	80	16	0	1
	B 2	1280	5120	640	200	64	1–2	1	640	2560	160	80	64	0	1
	B 3	640	640	160	80	16	0	1	640	640	40	10	16	0	1
	B 4	160	320	20	10	8	0	1	160	320	< 10	4	8	0	1
28	B 1	640	2560	320	200	32	1–2	1	640	2560	80	80	16	0	1
	B 2	640	5120	320	200	64	2	1	640	2560	160	80	32	0	1
	B 3	320	1280	160	80	32	1	1	320	640	80	20	16	0	1
	B 4	160	320	160	10	16	1	1	160	320	20	4	16	0	1
49	B 1	160	640	160	40	16	> 1	1	160	640	40	10	16	0	1
	B 2	160	1280	160	40	16	2	1	160	640	40	10	16	0	1
	B 3	320	1280	320	200	64	> 2	1	320	1280	160	80	64	0	1
	B 4	320	1280	320	200	64	1	1	320	1280	160	80	64	0	1

* 2-Mercaptoethanol reduction test. ssa = subsurface antigens. lps = lipopolysaccharide antigen.
† B 1; B 2: inoculated with *Br. abortus* strain 19. ‡ B 3; B 4: inoculated with *Br. abortus* strain 544.

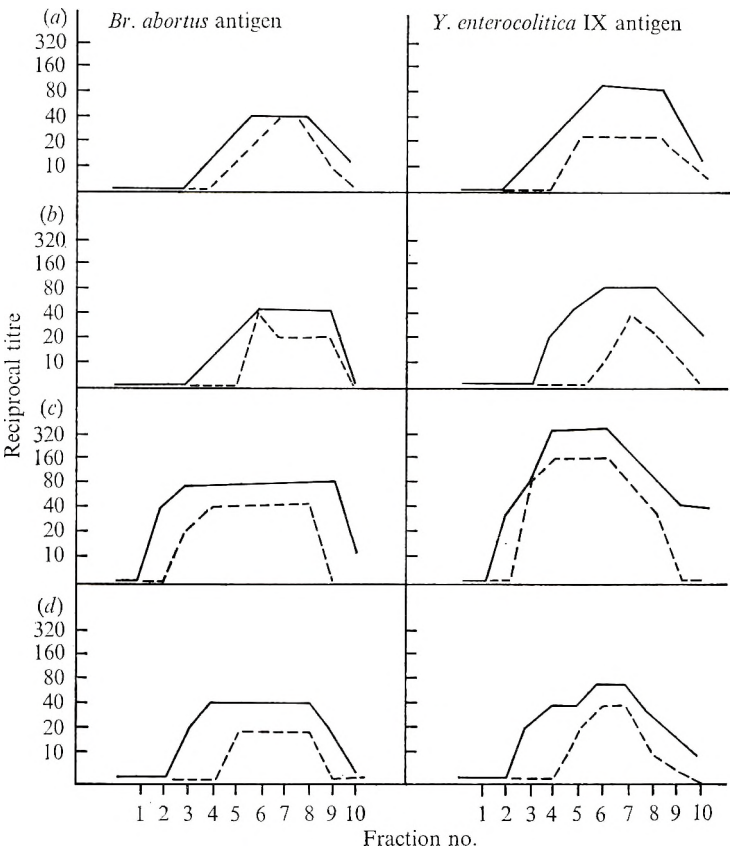

Fig. 1. Density-gradient centrifugation of bovine antisera to *Y. enterocolitica* IX. (*a*) Y 1, (*b*) Y 2, (*c*) Y 3, (*d*) Y 4, at 14 days post-inoculation. ——, SAT; – – – –, 2-Me.

(Tables 1, 2) showed that from the time of the RBPT becoming positive, antibodies to the two organisms could be differentiated on the basis of their titres to the two Rose Bengal stained antigens.

Characterization of the immunoglobulin classes of antibodies to the cross-reacting antigens of Br. abortus *and* Y. enterocolitica *IX*

Density-gradient centrifugation of serum collected from cattle at intervals after injection with *Y. enterocolitica* IX or *Br. abortus* gave results essentially consistent with those of the 2-mercaptoethanol tests. Thus both organisms initially evoked fast-sedimenting 2-mercaptoethanol-labile cross-reacting antibodies, which by the tenth day after inoculation were supplemented by slowly sedimenting 2-mercaptoethanol-stable antibodies. In *Y. enterocolitica* IX-inoculated cattle, the agglutinins remained of the slowly sedimenting type until they became undetectable (Fig. 1*a–d*). Similarly, in *Br. abortus*-inoculated cattle the agglutinins remained of the slowly sedimenting type but did not decline to undetectable levels in the period studied (Fig. 2*a–d*).

Ion-exchange chromatography of bovine antiserum to *Y. enterocolitica* IX

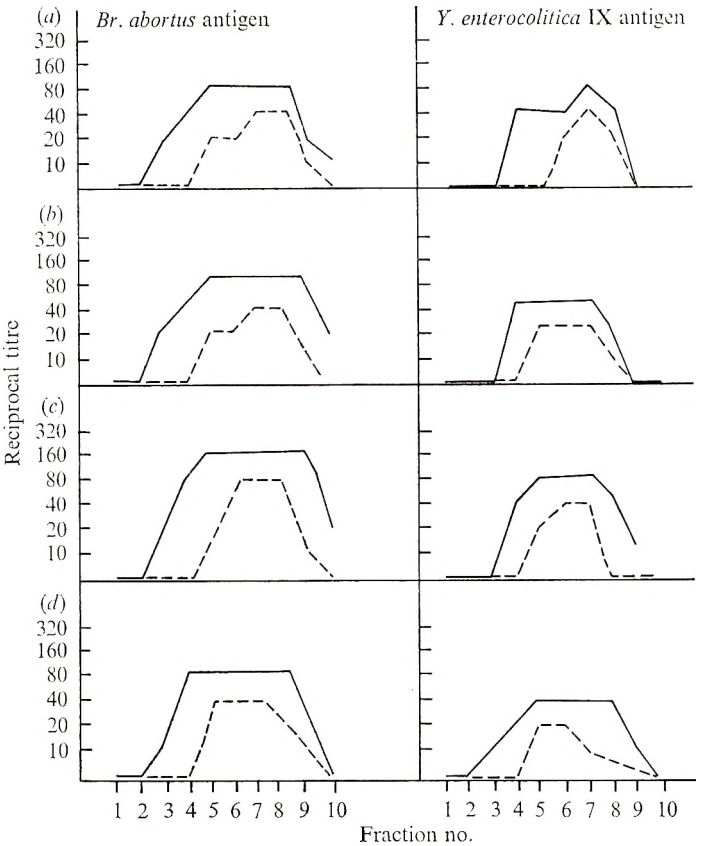

Fig. 2. Density-gradient centrifugation of bovine antisera to *Br. abortus* (*a*) B 1, (*b*) B 2, (*c*) B 3, (*d*) B 4 collected 49 days post-inoculation. ——, SAT; – – – –, 2-Me.

collected 14 days after inoculation showed that most of the 2-mercaptoethanol-stable agglutinating activity for *Br. abortus* was distributed between fractions 2 and 3, consisting mainly of IgG_1, with some IgA in fraction 3. Fractions 1, 2 and 3 contained most of the activity detectable in the Coombs antiglobulin test. An appreciable amount of agglutinating activity was eluted in fraction 5, which contained most of the serum IgM. This was largely susceptible to reduction with 2-mercaptoethanol (Table 3).

The results obtained on fractionation of bovine antiserum to *Br. abortus* collected 14 days after inoculation were qualitatively similar to those for the anti-*Y. enterocolitica* IX serum, with a high proportion of the agglutinins reacting with *Y. enterocolitica* IX being eluted in fractions 3, 4 and 5 (Table 3). The elution profiles for the two sera (Fig. 3) were essentially similar.

Attempts were also made to characterize the immunoglobulins involved in the cross-reaction by eluting antibodies absorbed by the heterologous organisms. No success was obtained with sera collected before the tenth day after inoculation, probably because of the low antibody concentrations present, but immunoglobulins were recovered in eluates from organisms treated with high-titre sera.

Eluates prepared from *Br. abortus* cells used to absorb antiserum to *Y. entero-*

Table 3. *Serological activity of fractions separated from bovine antisera to* Y. enterocolitica *IX and* Br. abortus *by ion-exchange chromatography on* DEAE-*cellulose*

		Reciprocal titres in tests with *Br. abortus* antigen							Reciprocal titres in tests with *Y. enterocolitica* IX antigen						
							Precipitins							Precipitins	
Serum	Fraction no.	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps
Y 3,	1	< 10	160	< 10	< 2	< 1	0	0	< 10	320	< 10	< 2	< 1	1	1
14 days	2	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	3	80	640	80	20	4	0	1	320	1280	320	20	32	2	1
	4	10	80	< 10	10	2	0	0	10	80	< 10	< 2	2	0	0
	5	20	80	10	< 2	< 1	0	0	40	160	10	< 2	< 1	0	0
	6	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	7	40	80	10	< 2	< 1	0	0	80	160	10	< 2	< 1	0	0
B 2,	1	< 10	640	< 10	< 2	< 1	1	1	< 10	640	< 10	< 2	< 1	0	1
14 days	2	< 10	10	< 10	< 2	< 1	0	0	< 10	10	< 10	< 2	< 1	0	0
	3	160	2560	160	200	64	2–3	1	160	1280	160	80	32	0	1
	4	10	80	10	20	8	1	1	10	80	10	10	8	0	1
	5	40	160	10	4	2	0	0	40	160	< 10	< 2	2	0	0
	6	< 10	< 10	< 10	< 2	< 1	0	0	< 10	< 10	< 10	< 2	< 1	0	0
	7	80	80	10	2	< 1	0	0	80	80	< 10	< 2	< 1	0	0

* 2-Mercaptoethanol reduction test. ssa = sub-surface antigens. lps = lipopolysaccharide antigen.

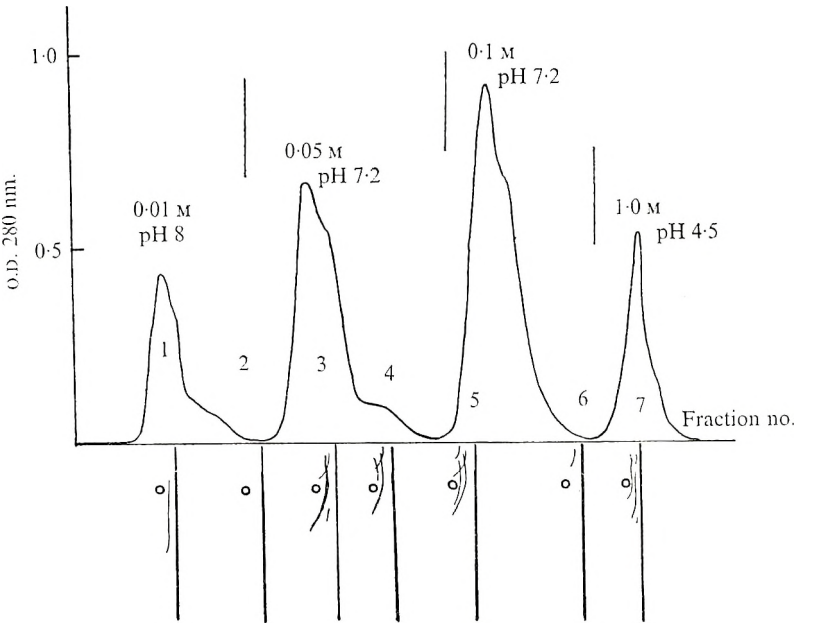

Fig. 3. Ion-exchange chromatography on DEAE-cellulose of bovine antiserum to *Y. enterocolitica* IX.

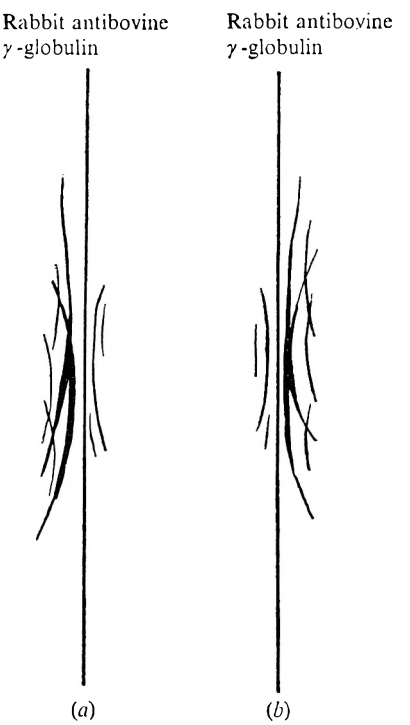

Fig. 4. (*a*) Immunoelectrophoresis of bovine serum (left) and eluate from *Br. abortus* cells used to absorb bovine antiserum to *Y. enterocolitica* IX (right). (*b*) Immunoelectrophoresis of eluate from *Y. enterocolitica* IX cells used to absorb bovine antiserum to *Br. abortus* (left) and bovine serum (right).

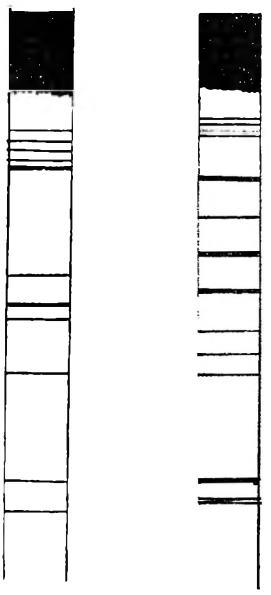

Fig. 5. Disk electrophoretic patterns of (*a*) eluate from *Br. abortus* cells used to absorb bovine antiserum to *Y. enterocolitica* IX. (*b*) eluate from *Y. enterocolitica* IX cells used to absorb bovine antiserum to *Br. abortus*.

colitica IX, contained immunoglobulins of IgA, IgG_1 and IgG_2 classes (Fig. 4*a*). IgM may have been present but was in too low concentration to be detectable by immunoelectrophoresis. A very similar immunoelectrophoretic pattern was obtained with eluates from the *Y. enterocolitica* IX cells used to absorb antiserum to *Br. abortus* (Fig. 4*b*).

Disk electrophoresis of the eluates confirmed their complexity (Fig. 5). The fast-migrating components were probably extracted from the bacterial cells by the acid buffer.

The anamnestic response to the cross-reacting antigen of Y. enterocolitica *IX*

Injection of cattle previously inoculated with *Y. enterocolitica* IX with a second dose of organisms after the initial antibody response had declined to insignificant titres, produced a rapid response with 2-mercaptoethanol-stable antibodies predominating at the sixth day. Precipitins and CF antibodies to both *Y. enterocolitica* IX and *Br. abortus* antigens were also detectable at this time. However, even though quite high antibody titres to *Br. abortus* were obtained, these declined rapidly as in the primary response (Table 4).

DISCUSSION

The nature of the serological response to the cross-reacting antigen of *Y. enterocolitica* IX was considered significant in relation to its possible effects on diagnostic tests for brucellosis. It was clear from both the present results and earlier studies,

Table 4. *The serological response of cattle previously inoculated with* Y. enterocolitica *IX to secondary stimulation with* Y. enterocolitica *IX cells*

		Reciprocal titres in tests with *Br. abortus* antigen							Reciprocal titres in tests with *Y. enterocolitica* IX antigen						
							Precipitins							Precipitins	
Day*	Sample	SAT	Anti-globulin (Coombs)	2-ME†	CFT	QRBPT	ssa	lps	SAT	Anti-globulin (Coombs)	2-ME*	CFT	QRBPT	ssa	lps
1	Y 1	10	20	< 10	< 2	< 1	0	0	40	40	< 10	< 2	1	1	0
	Y 2	20	40	< 10	< 2	< 1	0	0	40	40	10	< 2	1	1	0
	Y 3	40	40	10	4	1	0	0	80	80	10	< 2	2	> 1	0
	Y 4	80	160	< 10	< 2	< 1	0	0	80	80	< 10	< 2	2	1	0
6	Y 1	20	80	10	2	2	0	0	80	160	40	4	4	1–2	1
	Y 2	40	160	10	10	2	0	0	80	320	80	10	8	1–2	1
	Y 3	80	160	40	20	8	0	1	640	2560	320	20	32	2	1
	Y 4	160	320	40	20	8	0	1	640	2560	320	20	64	1–2	1
30	Y 1	20	40	10	< 2	< 1	0	0	80	640	40	< 2	1	1	0
	Y 2	20	80	10	< 2	< 1	0	0	80	320	40	< 2	2	1	0
	Y 3	40	160	20	< 2	1	0	0	160	640	80	4	8	2	1
	Y 4	40	80	20	2	2	0	0	320	1280	160	4	16	1	1

* Day after second inoculation with *Y. enterocolitica* IX cells. ssa = subsurface antigens. lps = lipopolysaccharide antigens.
† 2-Mercaptoethanol reduction test.

(Ahvonen *et al.* 1969; Corbel & Cullen, 1970; Hurvell *et al.* 1971; Akkermans & Hill, 1971) that, in all species studied, *Y. enterocolitica* IX evoked antibodies which superficially appeared indistinguishable from those provoked by infection with *Br. abortus*.

However, it was apparent from the present results that, although *Y. enterocolitica* IX could stimulate production of high titres of agglutinins to *Br. abortus*, it was relatively less effective in evoking CF and precipitating antibodies. Furthermore, unlike those provoked by *Br. abortus* infection, these cross-reacting antibodies were transient and rapidly declined to insignificant levels. Thus it was unlikely that *Y. enterocolitica* IX would cause an animal to become a persistent reactor to diagnostic tests for brucellosis. This meant that, with the present system of repeated tests, it would be unlikely to produce serious difficulty in the assessment of cattle for accreditation to brucellosis-free herds.

The nature of the antibodies produced in response to the cross-reacting antigen of *Y. enterocolitica* IX was clearly not significantly different in type from those evoked by *Br. abortus*. In both instances the fractionation results showed that 19 S IgM and 7 S IgG_1, IgG_2 and probably IgA antibodies were produced. This was not entirely unexpected in view of the similar serological activities which could be demonstrated in response to both antigens. However, as already indicated, the nature of the antibody response engendered by the agglutinogens of even closely related organisms cannot be predicted *a priori*. Thus the results of Smith *et al.* (1970) showed that the carbohydrate residues of lipopolysaccharide antigens, although largely determining serological specificity, did not determine the nature of the antibody response. Recent evidence (Lüderitz, Galanos & Rietschel, 1971) has suggested that the lipid A component may be significant in this respect.

Some evidence is available which suggests that brucella strains may vary in the pattern of the immunoglobulin response they elicit. Thus Howe (1970) observed that, in *Br. canis* infection in the dog, antibodies to the lipopolysaccharide antigen were almost entirely of the IgM class, whereas antibodies to the nucleoprotein and intracellular antigens were of IgG class. In *Br. suis* infections in man the antibodies produced against the lipopolysaccharide agglutinogen were predominantly IgG at all stages of infection (Howe, 1970). In *Br. abortus* infections in cattle, sequential production of IgM and IgG antibodies to the agglutinogen has been observed in both this and other studies (Rose, Lambert & Roepke, 1964; Rice, Tailyour & Cochrane, 1966). It appears from the present results that the cross-reacting antigen of *Y. enterocolitica* IX elicits a qualitatively similar response, the transient nature of which may be attributed to the minimal persistence of this organism. Ahvonen *et al.* (1969) noted that in human infections with *Y. enterocolitica* IX, the agglutinins cross-reacting with brucellas usually underwent rapid decline.

Although *Y. enterocolitica* IX has not been shown to infect cattle under natural conditions, it would appear that even should this occur persistent false positive reactions would not arise in the animals infected.

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The evidence for the airborne spread of Newcastle disease

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SUMMARY

Newcastle disease virus has been shown to survive when airborne in small particles, both in the laboratory and in the open air. Field outbreaks have been studied and viable virus has been recovered from the open air short distances downwind of infected premises. Vaccination of birds leads to a great reduction in the amount of virus liberated into the air.

INTRODUCTION

Newcastle disease is a highly infective and contagious disease of poultry. It causes outbreaks from time to time throughout the world and losses from the disease in Great Britain alone can amount to £20,000,000 annually.

Newcastle disease virus (NDV) has been classified in numerous ways (National Research Council, 1971), one of which designates the virus as pneumotropic, neurotropic or viscerotropic. These terms are subjectively derived and are based mainly upon the symptoms and lesions observed after natural or laboratory infections. The Asiatic form of the disease has been associated with visceral lesions in infected birds, rapid and very high mortality, and a low tendency to spread from farm to farm. Some laboratory strains such as Herts '33 are associated with symptoms in which paralysis and other neurological derangements predominate. Many British field-strains before 1970 were from outbreaks which showed a mixture of nervous and respiratory symptoms.

Field-studies of the present epizootic which started in August 1970 at Clacton on Sea showed that there was an unusual degree of respiratory distress followed by sudden death without the usual nervous symptoms, except in the later cases of the epizootic, when there seemed to be some modification of the disease. Although some cases were associated with enteric lesions these did not predominate, and in general the impression has been of a virus which affected the respiratory tract to an unusually high degree. This virus strain (designated Essex '70, was notable for the extremely rapid spread of the disease; since August 1970 there have been more than 7750 recorded outbreaks in Britain. At the same time disease caused by a virus later found to be of a similar nature occurred in Holland and

possibly in other countries in Western Europe. In the most recent comprehensive review of ND, Lancaster (1966) pointed out many ways in which the disease could be spread – for example, by the sale of infected carcasses, with subsequent infection of susceptible birds by the discarded unboiled giblets, or the movement of day-old chicks and point-of-lay pullets, subsequently known to have been in contact with the disease. In an area where the poultry industry is dense, both in the number of birds per site and in the total number of sites, the mechanism of spread may be obscured by the multiplicity of possible routes. These include the movement of supervisors and other itinerant employees, of birds, feed lorries, packing station crews and the free movement of sparrows and other wild birds. Lancaster also pointed out some experimental data suggesting evidence of airborne spread, by Idani & Seetheraman (1947) and by Delay, Deome & Bankowski (1948). Andrewes & Allison (1961) showed that in laboratory experiments cross-infection with NDV could take place via the air, but suggested that, because the effective distance in their experiments was short (less than 9 in), the virus was either carried in large particles which would not travel beyond this distance, or it was carried in small particles in which the virus was very labile. They also showed that there appeared to be a period of maximum infectivity, during the last 36 hr. of the infection. The air-spray technique of administering vaccine also shows that the virus will survive in the air, at least for short periods. Outbreaks during 1960–2 were shown by Smith (1964) probably to have been windborne as they followed the expected downwind deposition pattern of Pasquill's (1961) formulation.

The persistence of ND, and the ease with which it has spread in the recent outbreak, have been attributed to the high resistance of the virus to adverse conditions. This has raised once again the suggestion that some of the spread of the disease is by the airborne route. Therefore a series of experiments was carried out to obtain information on the following points:

(*a*) Do infected birds excrete virus into the air and, if so, when does the maximum output occur?

(*b*) What is the influence of relative humidity on the survival of the airborne virus?

(*c*) Can the virus remain infective in the open air?

(*d*) Can the virus be recovered from the air inside infected premises and also in the open air downwind of such premises?

(*e*) Does vaccination influence the excretion of virus from infected birds?

Information on all these points might be of considerable importance in the design of effective control measures.

MATERIALS AND METHODS

Organisms

The three strains of NDV used were Herts '33/56, Eastwood '67 and Essex '70. Suspensions of these strains were prepared at the Central Veterinary Laboratory, Weybridge (CVL), by harvesting the allantoic fluid from infected embryonated eggs two days after infection. These were stored at $-70°$ C.

Bacillus subtilis var. *niger* spores (BG) were prepared as thick (approx 10^{11}/ml.) suspensions which were stored at 4° C. Just before use the small quantity required for a test was heated at 60° C. for 30 min. to eliminate any germinated spores or vegetative forms.

Escherichia coli (*EC*) MRE Strain No. 162 was grown in aerated culture vessels in tryptone broth containing glycerol (Elsworth *et al.* 1968).

Laboratory tests with aerosols

Preparation of suspension for spraying. The required quantity of heated BG suspension was centrifuged and the supernatant was removed from the pellet, which was then resuspended in the virus suspension. The final concentration of BG was approximately 10^{10}/ml.

Generation and storage of airborne particles. Aerosols were generated by spraying the virus suspensions containing BG with a Collison atomizer in a Henderson apparatus modified to operate over a range of relative humidities (Druett, 1969). Clouds were sampled immediately after generating (cloud age about 1 sec.) and after storage in the dark in a 500 l. rotating stainless-steel drum (Goldberg, Watkins, Boerke & Chatigny, 1958). Samples were collected from the storage drum after holding the cloud for 30, 60 and 240 min.

Cloud sampling. Samples were collected for 1 min. with raised impingers (May & Harper, 1957) at a flow rate of 11 l. per min. The collecting fluid was 10 ml. of nutrient broth containing 200 units of penicillin G.

Exposure to the open air

The microthread technique of May & Druett (1968), in which the airborne organisms are captured and held on ultrafine spider threads, was used. In many previous tests this method has been shown to give results similar to those found with airborne organisms. Microthreads were loaded with organisms by passing the cloud generated in the Henderson apparatus, as used for the laboratory tests, through sets of frames held in 'sows' (May & Druett, 1968). The exposure of the loaded microthreads was carried out in a ventilated unit designed by Druett (1971). This avoids the possibility of any virus particles that might be detached from the microthreads during exposure becoming airborne in the open air. In this unit the microthreads were exposed by pulling the open air through the exposure section at *ca.* 0·8 m./sec. The air was then passed through a high-efficiency filter before being discharged to the atmosphere. All the manipulations of the microthreads, once they were loaded with virus, were carried out in a safety cabinet vented via a high-performance filter.

Viability assessment

Bacteria. Suspensions, cloud and microthread samples were diluted in phosphate buffer (Anderson, 1966). The surface of each of four plates of tryptone agar was inoculated with 0·25 ml. volumes of the appropriate dilution. EC and BG colonies were counted after incubation of the plates overnight at 37° C.

Virus. NDV was assayed, usually immediately after collection, by inoculating

0·1 ml amounts of suitable dilutions in nutrient broth into the allantoic fluid of 9-day fertile eggs. The presence of virus in the inoculated eggs, after death of the embryo, was checked by haemagglutination tests. The virus titres were obtained by using the Spearman-Karber method (Finney, 1964).

Determination of viability of airborne organisms generated in the laboratory. The concentration of viable organisms in clouds or on microthreads is reduced by both physical loss and viable decay. To study loss of viability it is necessary to estimate the physical loss. The method used is to mix the bacteria or virus under test with a non-decaying tracer organism, BG. This will be subjected to the same physical loss as the test organism. The ratio of viable test organisms to viable BG in the starting suspension is equated to 100 % viability and the ratio of test organisms to BG in the cloud or microthread samples is expressed in terms of this ratio.

Air sampling

The Litton Large Volume (LV) air sampler (Litton Industries, Minneapolis, Minnesota, U.S.A.), which will concentrate particles from large volumes of air into a small volume of liquid, was selected for use, as very small numbers of virus particles were expected in the air. To obtain the maximum concentration of airborne material, it was necessary to recirculate the collecting fluid in the air sampler for periods up to 60 min. In this way the particles in 60,000 l. of air could be concentrated into about 30 ml. of fluid. Before using this technique it was necessary to know whether this long recirculation could cause any loss of viability to NDV collected early in the sampling period. To test this, a known quantity of Herts '33 strain virus was recirculated in the Litton air sampler. This was run in a virus-free atmosphere for 60 min. Samples were removed at intervals of 15 min. and these were titrated for NDV. There was no significant loss of virus during the 60 min. of running time.

Measurement of particle size

(*a*) *Cascade impactor* (May, 1945). Clouds were sampled at a flow rate of 17 l./min. onto slides coated with 5 % (w/v) gelatin in 10 % glycerol using a cascade impactor. The collected organisms were recovered by dissolving the gelatin–glycerol film in phosphate buffer at 37° C.

(*b*) *The multistage liquid impinger* (May, 1966). This sampler collects at 55 l./min. and is useful where the virus concentration is expected to be low. The particle-size distribution is as follows: top stage > 6 μm., middle stage 3–6 μm. and bottom stage < 3 μm. The collecting fluid was distilled water containing antibiotic.

RESULTS

Excretion of virus into the air by infected birds

These experiments were carried out using two strains of NDV. In the first experiment, five 6-week-old white Leghorn growers were inoculated intravenously with $10^{5\cdot3}$ ELD 50 of the Herts '33/56 strain and these donor birds were placed with 15 similar but uninoculated birds. These birds were held in a room 10 × 10 × 10 ft.,

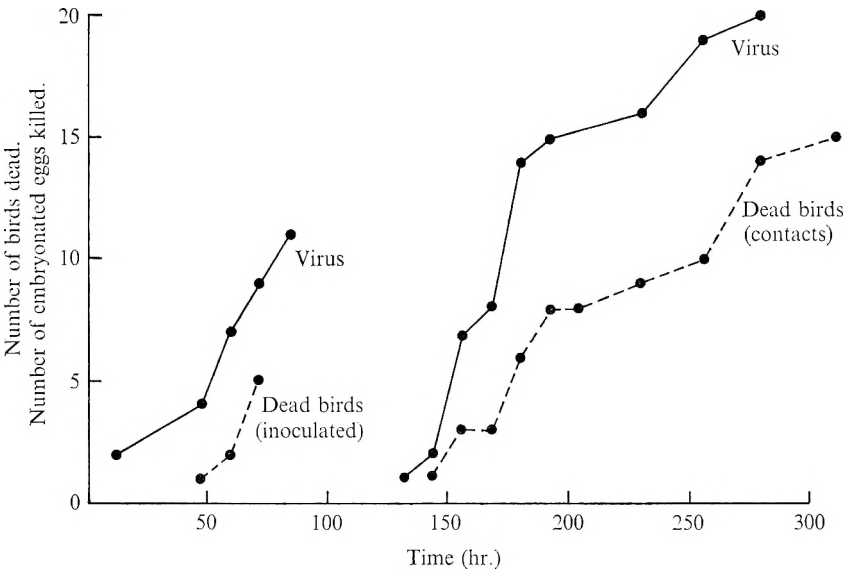

Fig. 1. Bird mortality and airborne virus recovered from contact experiment. (Virus concentration is expressed as the cumulative number of embryonated eggs killed when inoculated with 0·1 ml. (≡ 200 l. air) of fluid from the Litton Air Sampler.)

with positive pressure ventilation and a filtered exhaust system, maintained at 16° C. The relative humidity (R.H.) was between 30 % and 60 %. A chicken-wire partition, 6 ft. high, restricted the birds to two-thirds of the floor area. The Litton sampler was placed outside this partition at a height of 1 m. To reduce the amount of bacterial and spore contamination that would be generated from the normal litter, the floor area was covered with brown paper, which was replaced daily. This also reduced the chance of generation of airborne virus from the droppings. For the first 9 days of the experiment the air was sampled twice daily, starting at 09.00 and 20.00 hr. Thereafter the sampling was carried out only once per day. The Litton Sampler was primed with approximately 30 ml. of peptone–water broth containing penicillin G 5000 units/ml. and Fungizone* 20 μg./ml., which was recirculated through the sampler during the 60 min. sampling period. After each sampling period the sampler was removed from the room and cleansed with distilled water containing 1 % BRIJ 35 (non-ionic detergent, B.D.H. Ltd) and then washed with sterile distilled water. Dead birds were removed from the room at regular intervals.

In the second experiment, using the field strain Essex '70, 20 six-week-old Light Sussex birds were used. The air was sampled only twice, when the in-contact birds showed signs of disease, at 188 and 238 hr. after the start of the experiment.

Fig. 1 shows the results of the first experiment. The virus concentration is expressed as the number of embryonated eggs killed when inoculated with 0·1 ml. of the sample fluid from the Litton sampler, containing the particles from approximately 200 l. of air. As only small quantities of virus were recovered, it

* Amphotericin B, E. R. Squibb & Sons, N.Y., U.S.A.

was not possible to estimate the virus content more precisely. Nevertheless it is obvious that virus is present in the air and that it is present at least 12 hr. before the death of the birds. The in-contact birds, which are presumably infected from the airborne virus released from the inoculated donor birds, started to die about 100 hr. after the release of virus from the donor birds. There is probably a similar effect in the last days of the experiment: the last five birds to die were probably infected with virus excreted by the first in-contact birds to show infection.

The second experiment showed that the Essex '70 strain of virus killed birds more rapidly. All 15 in-contact birds were dead after 238 hr. The virus concentration in the air samples was higher and estimations of virus titres by the method of Spearman & Karber (Finney, 1964) was possible. At 188 hr. 0·1 ml. of air-sampler liquid contained $10^{2\cdot45}$ ELD 50 and at 238 hr., when all the birds had died, it was $10^{1\cdot05}$ ELD 50. It was noted that the Light Sussex birds used in this experiment tended to huddle more than the white Leghorns used in the previous experiment with the Herts '33/56 strain.

The influence of relative humidity on the survival of airborne virus

Having established that the NDV could be recovered from the air of rooms containing experimentally infected birds, where the relative humidity (R.H.) was controlled between 30 % and 60 %, it was desirable to investigate the effect of a wider range of R.H. on the survival of airborne virus, as in commercial poultry houses the R.H. will vary widely from time to time. Three strains of NDV were used: Herts '33/56, Eastwood '67 and Essex '70. Aerosols of the three strains were generated and stored in the dark, at a variety of R.H. values, in a rotating drum and sampled as described in Methods.

As the suspensions of virus appeared to be much more viscous than the suspensions of bacteria usually used in this apparatus, it was first necessary to establish the size of the particles produced when allantoic fluid was sprayed from a Collison atomizer. Therefore clouds were generated from allantoic fluid containing BG in the Henderson apparatus and these were sampled approximately 1 sec. after generation, using a cascade impactor. These samples showed the following particle size distribution:

	Size limit of particles impacted (μm.)	% of total organisms recovered
Stage 1	6·0–2·0	0·3
Stage 2	2·2–6·0	5·2
Stage 3	1·0–3·0	35·7
Stage 4	0·5–1·5	58·8

Most of the particles generated were in the range of 0·5–3·0 μm similar to those usually found when aqueous suspensions of bacteria were used in this apparatus.

The results obtained using the three strains of NDV at various R.H. values are shown in Table 1. With all three strains, 30 % or more of the virus survived the initial spraying process. All three strains showed that 1 % or more of the virus remained viable after 4 hr. With the Essex '70 strain at 80 % R.H. viable virus was

Table 1. *Viability of three strains of NDV after holding in the rotating drum at 20° C. for various times and at different relative humidities* (R.H.)

Virus strain	R.H.	% viability after holding for 30 min.	60 min.	240 min.
Essex '70	80	36	12	6
	70	49	95	15
	60	40	33	9
	50	13	7	2
Herts '33/56	80	96	53	11
	80	67	—	11
	70	88	47	7
	60	18	14	3
	50	21	23	3
Eastwood '67	80	40	17	5
	70	64	38	10
	60	111	52	9
	60	34	39	4
	50	2	1	1

recovered after holding the cloud for 16 hr. but there were insufficient data to calculate accurately the amount present. Survival of all three strains at 50 % R.H. was lower than at 60–80 % R.H. There was insufficient evidence to show that any of the three strains survived better than the other two, under the conditions of this test.

The effect of exposure to open air on the infectivity of the virus

As an extension of the studies of survival of NDV in the rotating drum, experiments were carried out with virus particles held on 'microthreads'. It has been shown by Druett & May (1968), and by May, Druett & Packman (1969), that the toxicity of the open air varies from time to time when tested with a variety of micro-organisms exposed on microthreads. This germicidal property of the open air was attributed to the presence of an unstable Open Air Factor (OAF) which was rapidly lost when the air was enclosed. As an indicator for the toxicity of the open air, a reference micro-organism was included in our tests. The reference micro-organism, EC, has been used extensively for measuring the toxicity of the air and is considered to be an aerosol-robust micro-organism.

In the tests reported here the results obtained with three strains of NDV and the reference strain of EC are shown in Table 2. The survival of NDV was usually similar to that of EC exposed at the same time, indicating that NDV was not unduly sensitive to OAF. There was, as with the previous experiment, an initial loss of viable virus (approx 50 %), similar to that found with many other micro-organisms. For ease of presentation, the initial unexposed samples, collected at time 1 sec., have been given a nominal viability value of 100 %. Though it was not possible to select exactly similar conditions for comparing the different strains, a reasonable range of wind directions and R.H. values was used in tests with each of the strains. With all three strains, a significant quantity of virus survived for at least 30 min under all the conditions encountered.

Table 2. *The viability of three strains of NDV held in the open air at various relative humidities* (R.H.) *and in winds from various directions*

(The control at 0 min. was given the nominal value of 100 %. The viability of *E. coli* 162 (EC) exposed at the same time is given for comparison.)

		Wind			Time of exposure		
Strain	R.H.	Direction	Speed (m./sec.)	Organism	30 min.	60 min.	Control, 60 min.
Essex '70	60	040°	25·5	NDV	62	31	36
				EC	26	8	79
	63	340°	17	NDV	24	8	4
				EC	10	3	84
	53	250°	20·4	NDV	20	0	18
				EC	9	3	41
	62	245°	17	NDV	22	5	28
				EC	6	3	74
	93	120°	8·5	NDV	11	0	46
				EC	5	0	82
Herts '33/56	75	290°	17	NDV	23	23	36
				EC	29	42	39
	95	190°	46	NDV	48	7	6
				EC	43	21	93
	70	300°	36	NDV	7	11	52
				EC	34	8	63
	78	070°	8·8	NDV	5	0	35
				EC	1	2	70
Eastwood '67	63	050°	18·8	NDV	30	—	38
				EC	3	—	76
	60	270°	18·8	NDV	212	59	189
				EC	20	2	103
	65	220°	24	NDV	1	< 1	4
				EC	28	13	81
	65	270°	27·2	NDV	51	3	9
				EC	36	6	130
	68	070°	13·6	NDV	5	0	35
				EC	6	1	68

Recovery of virus from the air inside and outside infected premises

Three farms at which there were reported outbreaks of Newcastle disease were used. Farm A (see plan, Fig. 2) consisted of eight poultry houses, each of which had been stocked with 8500 1-day-old chicks on 27–29 October 1970. On the day of sampling, 16 November 1970, 4000 birds were reported to be sick in the third house. On arrival at the farm a meteorological station was set up upwind of the houses. This recorded continuously the wind direction and speed, and air temperature, at a height of 2 m. above ground level, during the time that the air samples were taken, in this instance at night. Air sampling with a Litton LV sampler collecting at a rate of 1000 l./min. into 28 ml. of fluid was carried out, for

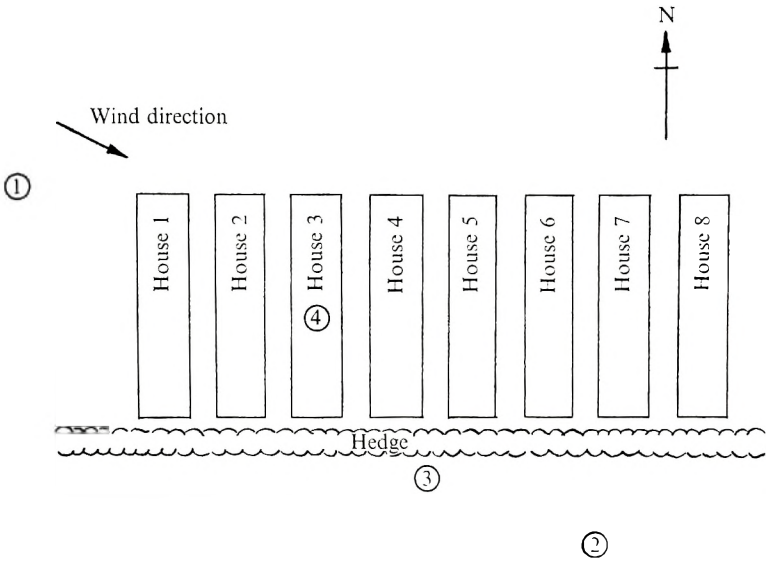

Fig. 2. Sampling sites (○) on farm A (not to scale).

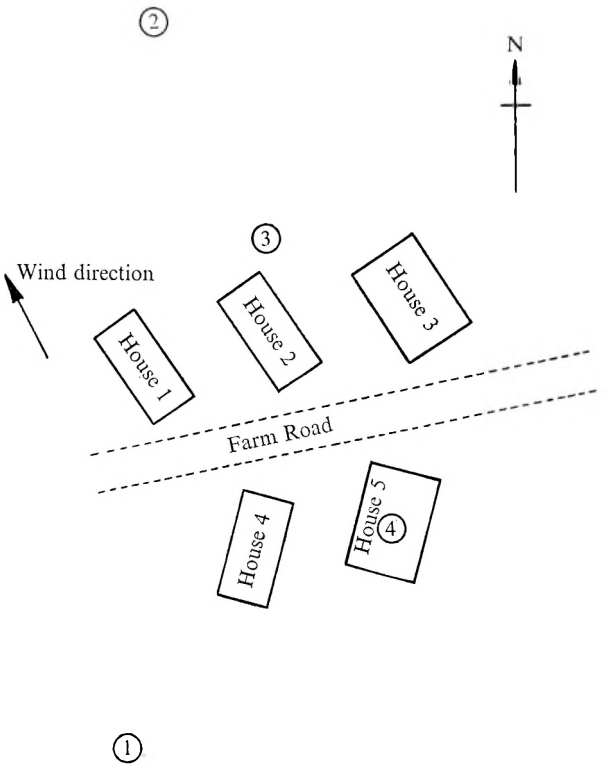

Fig. 3. Sampling sites (○) on farm B (not to scale).

Table 3. *Amount of virus recovered from samples taken at various positions in and around two farms where there were outbreaks of Newcastle disease*

Sample position	Site no.*	R.H.	Temp. (°C.)	Virus recovered†
Farm A. (Samples taken at night, wind speed 0·4–0·7 m./sec., 280–295°; 8/8 cloud.)				
Upwind (21 m.)	1	94	6·1–6·9	Trace‡
Inside house	4	67	21·0–21·8	5·7
Downwind (23 m.)	3	96	4·2–4·4	2·95
Downwind (55 m.)	2	92	5·3–5·5	2·0
Farm B. (Samples taken in daylight, wind speed 2·9–3·9 m./sec., 130–145°; 6/8–8/8 cloud)				
Upwind (101 m.)	1	82	8·3	Trace‡
Inside house	4	70	15–15·5	5·28
Downwind (64 m.)	3	79	8·3–8·6	1·45
Downwind (165 m.)	2	84	7·8–8·3	Nil

* See Fig. 1.
† Virus titre, $\log_{10}$ ELD 50 per 33,000 l. of air.
‡ Trace = too low for accurate estimation.

30 min. each at (1) upwind of the poultry houses beside the meteorological station, (2) 55 m. downwind of the farm buildings (as far as it was possible to go without encountering obstructions between the farm building and sampling station), (3) 23 m. downwind of the building containing the infected birds, and (4) inside the house containing the infected birds. The height of the sampler above ground level was 1·7 m. when used in the open air and 0·5 m. when used inside the building. After sampling, the collecting fluid was drained off and stored at 4° C. before assessment. The machine was then flushed out with 0·1% BRIJ 35 solution followed by sterile distilled water and then refilled with collecting fluid.

At the second farm, B (Fig. 3), houses (1) and (2) had been stocked on 5 October 1970 with 7500 1-day-old chicks, house (3) with 13,000, house (4) with 9000 and house (5) with 14,000. Excess deaths were first noticed in houses (3) and (5) on 13 November; by 17 November, when the air samples were taken during daylight, 11,000 birds had died in house (5) and the moribund and morbid birds were showing signs of respiratory distress. Air sampling was carried out as on farm A. The sampling sites and their distances are shown in Fig. 3 and Table 3. At this farm the sample taken inside the building (sample (4)) was taken in two parts: (1) for 10 min. followed by (*b*) a 20 min. sample, as it was thought possible that the very high level of ammonia in the air might have a deleterious effect on the virus being circulated in the sampling fluid. At both farms the inside samples were taken with the ventilating fans off and the outside samples with the fans on.

The results of the virus recoveries in samples taken at farm A are shown in Table 3. Sample (4), taken inside the infected house, contained $10^{5·7}$ ELD 50 virus units in 33,000 l. of air, samples (2) and (3), collected downwind of the poultry houses, also contained significant amounts of virus. The sample taken upwind of the premises (sample (1)) contained a very small amount of virus insufficient for accurate estimation. It is possible that, as the sampler had been previously used

Table 4. (*a*) *The size distribution of the particles carrying Newcastle disease virus recovered from air inside a poultry house at farm C*

Particle size (μm.)	Stage of multistage sampler	Virus recovered*	
		At litter level	1·7 m. above floor
> 6	Top	3·39	3·11
3–6	Middle	3·14	3·31
< 3	Bottom	1·68	1·97

* Virus titre = $\log_{10}$ ELD 50 per 1000 l. of air.

Table 4. (*b*) *The size distribution of particles carrying bacteria recovered from air inside normal poultry houses*

Particle size (μm.)	Stage of multistage sampler	% of total bacteria recovered (at litter level)	
		House 1	House 2
> 6	Top	94	83
3–6	Middle	5	11
< 3	Bottom	1	6

earlier in the day at CVL, this small amount of NDV could be a carry over caused by insufficient washing of the sampling apparatus.

Similar amounts of virus were recovered from air samples at farm B (Table 3). Once again the upwind sample contained a small amount of virus, thought to be due to contamination. The downwind sample, collected 165 m. away from the infected premises, did not yield any NDV. As this sample was collected during an overcast day, the lack of viable virus in the sample might have been due to the virucidal effect of daylight.

At farm C the houses had been stocked on 3 January 1972 with 12,750 1-day-old chicks. These had been vaccinated on arrival with infectious bronchitis vaccine. On days 3 and 14 they received the vaccine strain Hitchner B1 virus in their drinking water, and on days 28 and 31 La Sota virus was also given in their drinking water. Air samples were taken on day 37 when the birds were dying in large numbers. By the end of the following week when the outbreak was over, some 40 % of the birds had died. At this farm the air was sampled using a multistage liquid impinger (May, 1966), collecting at 55 l./min. in an attempt to estimate the particle size of the airborne virus. Air samples were collected for 30 min. inside the building with the fans off: sterile distilled water containing penicillin–streptomycin (200 units/ml.) was used as the collecting fluid. Immediately after sampling the fluid from each stage was recovered and stored at 4° C.; it was titrated immediately on returning to the laboratory 4·5 hr. later. Virus recoveries are shown in Table 4(*a*); the quantities of virus recovered when sampling at litter level and at 1·7 m. above the floor level are similar. It can be seen that nearly all of the virus is collected in the top two stages, equally divided between particles from 3–6 μm. and those above 6 μm. Comparable concentrations of virus were recovered from

Table 5. *Comparison of the amounts of NDV recovered from vaccinated and non-vaccinated birds*

No. of days after challenge		Birds		Virus recovered*
		Live	Dead	
4	Vaccinated	90	0	2·72
	Non-vaccinated	86	4	4·66
5	Vaccinated	90	0	2·24
	Non-vaccinated	47	43	4·18
6	Vaccinated	90	0	2·20
	Non-vaccinated	23	67	2·87

* Virus titre, $\log_{10}$ ELD50 per 1000 l. air.

the air at this farm using the multistage sampler as at the other two farms when the Litton Sampler was used. Some air sampling experiments (unpublished work), carried out in normal non-infected poultry houses, showed that the bacterial flora of the air in a house was carried mostly on the larger particles (Table 4*b*).

The influence of vaccination of infected birds on excretion of virus into the air

Two batches of 90 Ross birds were used. They were kept in separate environmentally controlled rooms with a volume of approximately 1000 ft.^{3} each. Batch A birds were inoculated with La Sota virus in their drinking water when 21 and 35 days old. Batch B birds were not vaccinated. When both batches of birds were 56 days old they were each challenged with $4 \times 10^{10 \cdot 6}$ ELD50 Essex '70 virus, diluted to 25 ml. with distilled water and sprayed from a Humbrol aerosol propellant generator. From the 4th to the 6th day after challenge, the air in both holding rooms was sampled each day for 30 min using a multistage liquid impinger. The ventilating fans were turned off during the sampling period. The collecting fluid was distilled water containing streptomycin, and the samplers were at floor level. The fluid was recovered directly after sampling and titrated immediately. The results are shown in Table 5 and it is obvious that a much larger amount of virus is excreted by the non-vaccinated birds. The amounts of virus recovered per bird per hour, based on the number of birds alive during sampling, were about the same for each day with the vaccinated birds. For the unvaccinated birds it fell for the last day, but there was still more virus excreted at this stage than from the vaccinated birds. There were a number of non-specific deaths in the eggs used for NDV titration during the first part of the experiment: this was overcome in later samples by increasing the concentration of streptomycin in the collecting fluid. Although these non-specific egg deaths affected the precision of the titration, the benefits of vaccination were so obvious that the conclusions were not affected.

DISCUSSION

The results of laboratory experiments and field sampling reported here make a strong case for implicating the airborne travel of viable Newcastle disease virus

(NDV) as a means of spreading the disease during large outbreaks of Newcastle disease such as we have recently encountered in Great Britain.

A preliminary laboratory experiment with the Herts '33/56 strain, in which an attempt was made to reduce the amount of possible regeneration of virus from bird droppings by replacing the usual straw or sawdust litter with paper sheets, showed that virus could be recovered from the air in poultry houses containing experimentally infected birds. Although only small quantities of virus were recovered, it was obvious that it was excreted into the air at least 12 hr. before the first deaths from the disease. The in-contact birds started to die about 100 hr. after the virus was first found in the air, and the amount of virus recovered was in proportion to the number of infected birds. A second experiment using the Essex '70 strain of virus (isolated in the recent outbreak in U.K.) gave similar results, but in this case the progress of the disease was more rapid and the amount of virus recovered from the air was greater. At 188 hr., when the first infected birds died, the virus concentration was $10^{2 \cdot 45}$ ELD 50 in approximately 200 l. of air.

Once it had been established that viable virus was excreted into the air, it was necessary to carry out some investigations of the survival of the virus within the range of temperature and R.H. commonly encountered in Britain. These experiments were carried out in a closed container. Because of previous findings that there were present in the open air certain toxic factors capable of reducing the viability of airborne micro-organisms, they were followed by a series of tests where the virus was exposed on microthreads to the open air. This method has been shown to give results similar to those obtained with cells carried on free airborne particles. Although the range of meteorological conditions could not be selected in advance, exposures were carried out in a variety of conditions that might be expected after dark in Britain. From the results of these experiments it was obvious that the virus could survive for a sufficient length of time to make the airborne spread of the disease possible.

Having established that the virus could survive in the open air it was desirable to attempt to recover viable virus from the air in the vicinity of infected premises. On these occasions, sampling apparatus was taken to farms where outbreaks of Newcastle disease had been reported. Samples of air were collected inside the infected houses and at two of the farms samples were also collected outside at distances up to 165 m. downwind of the premises. Appreciable quantities of virus were recovered from the air inside the houses at all three farms and less but still measurable quantities at distances up to 64 m. downwind. The sample taken at 165 m. downwind at farm B yielded no virus, but it should be remembered that this sample was collected during daylight.

Finally, an attempt was made to investigate the effect of vaccination on the output of airborne virus from infected birds. From the results it is clear that a much larger amount of viable virus is excreted into the air by the unvaccinated birds. The protection conferred by the vaccine is obvious, but nevertheless it is significant that the protected infected birds did excrete quite appreciable quantities of virus into the air.

Though fairly conclusive, these investigations pose many extra questions. For

instance how does the size of the particle in which the virus is airborne affect its survival? It has been shown (Zalko-Titanenko, 1965; May, 1966) with other micro-organisms that viable decay of airborne cells occurs more rapidly in small particles than in large ones. Where do the particles of different size lodge in the respiratory tract of birds? With other animals it has been shown that the size of the particle carrying airborne micro-organisms is of importance in determining where they lodge in the respiratory tract and the number of organisms needed to initiate infection by the respiratory route (Druett, Henderson, Packman & Peacock, 1953). The respiratory tract of birds is quite different from that of other animals and there seems to be no information on penetration of particles into it, or the number of NDV particles needed to infect by this route. Our investigations indicated that most of the airborne virus was carried in particles exceeding 3 μm. in diameter. Is this the size of the particles excreted or is it a secondary aerosol after absorption onto particles of feather or litter? Is the particle size of the excreted virus affected by vaccination? What is the optimum particle size for the economical and efficient distribution of airborne vaccine?

Studies along most of these lines are now being planned or are already in progress.

We wish to thank Mr M. Andrews and Mr G. Parsons for their technical assistance.

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Staphylococcal infection in an intensive-care unit, and its relation to infection in the remainder of the hospital

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SUMMARY

A survey of the staphylococcal infections occurring in a general hospital over a period of four and a half years showed that multiple-resistant strains of phage type 77 were endemic in the medical and surgical wards. Strains of this phage type were uncommon among patients attending the casualty department, and those found were usually either fully sensitive to antibiotics or resistant to benzylpenicillin only. Regular monitoring of patients admitted to the intensive-care unit showed that 58 % of staphylococcal infections in such patients were present at the time of admission to the unit. Although the wards thus constituted a significant reservoir of infection for the intensive-care unit, there was no evidence to suggest that the return of patients from the unit to the wards was responsible for the transfer of infection in the opposite direction. The possibility of reducing the numbers of multiple-resistant staphylococci in the general wards, by the screening of all new admissions for the presence of tetracycline-resistant strains, appears to be impracticable in this area.

INTRODUCTION

The problems created by staphylococcal infections occuring in general surgical wards are well known, and have received considerable attention. Much less consideration has been given to the study of such infections occurring in intensive-care units, where the emphasis has largely been on the epidemiology of infections caused by Gram-negative bacilli. However Rountree & Beard (1968), studying a two-bedded unit, found that 20 out of 22 tracheostomies performed in the course of a year became colonized by *Staphylococcus aureus*. Jensen & Lassen (1969), recording 32 cases of staphylococcal tracheostomy colonization, found that in 11 of the patients the staphylococci responsible were resistant to methicillin. Both groups of workers emphasize the unique opportunities for dissemination inherent within an intensive-care unit, but less account is taken of the effect which such a unit may have on the incidence of staphylococcal infection elsewhere in the hospital.

In theory the temporary aggregation of susceptible patients in a unit where there may be numerous opportunities for spread of infection, and where there is extensive use of antibiotics leading to the selection of resistant strains of bacteria, could lead to the establishment of a reservoir of infection. Patients who acquired

multiple-resistant staphylococci in the intensive-care unit might carry these with them when they no longer required the special facilities and were returned to the general wards.

In this hospital, over the period 1968–71, there has been a high incidence of methicillin-resistance among strains of *Staph. aureus* isolated from infective lesions of in-patients, the majority of the resistant strains belonging to phage type 77. An intensive-care unit had been opened in the hospital in June 1967, and a preliminary survey (Harris, 1970) indicated virtually no difference in the incidence of methicillin-resistance between strains of *Staph. aureus* isolated within the unit and those isolated from the general wards of the hospital in the period ending July 1969. However, it was felt worth while to make a more detailed study of the interrelationship (in terms of both phage types and antibiotic sensitivities) of staphylococcal infections occurring in the intensive-care unit, in the general medical and surgical wards, and in the out-patient community. A limited amount of information was also obtained on the nasal carriage of staphylococci by nursing staff. This study, which extended over the period June 1967 to December 1971, forms the basis of the present report.

METHODS

Nature of the unit, and submission of specimens

The five-bedded intensive-care unit in question has already been described in detail, together with particulars of the methods routinely employed to limit cross-infection (Harris, Orwin, Colquhoun & Schroeder, 1969). It caters primarily for the treatment of respiratory insufficiency, but also undertakes the monitoring of cardiac arrhythmias following myocardial infarction. More detailed information on the type of cases treated has been published elsewhere (Colquhoun & Harris, 1971). Patients may be admitted either directly from the community via the casualty department or transferred from the general wards. Very few patients are admitted from the wards of other hospitals. Respiratory care is given by members of the anaesthetic staff, but in other respects patients remain under the supervision of general physicians and surgeons. Specimens for laboratory examination (sputum, urine, swabs from tracheostomies and other wounds) are submitted where appropriate, on the patient's admission to the unit and thereafter twice weekly for the duration of his stay.

Bacteriological methods

Specimens were examined by conventional cultural techniques. Sensitivity tests (for all antibiotics except methicillin) were carried out by a disk-diffusion method which has been described in full elsewhere (Harris, 1970). Sensitivity to methicillin was determined by inoculating a broth culture of the staphylococcus on plates containing 10 μg. methicillin per ml. which were incubated at 30° C. The Oxford staphylococcus was used as control organism. For the purposes of this study, a special record was kept of the sensitivity of *Staph. aureus* to the following antibiotics: benzylpenicillin, methicillin, streptomycin, tetracycline,

erythromycin and kanamycin. All strains of coagulase-positive staphylococci isolated in this laboratory are phage-typed, employing the standard phages, methods and criteria of the Central Public Health Laboratory, Colindale (Williams & Rippon, 1952; Blair & Williams, 1961). In this study, strains from any given patient which possessed the same phage-type and antibiotic-sensitivity pattern were considered to be identical. Strains differing in phage-type or in sensitivity to at least two antibiotics were regarded as representing distinct infections. During the period under review, 111 strains were isolated from 103 patients.

Accumulation of data on other patients and nursing staff

A record of the phage type and antibiotic sensitivity was available for all strains of *Staph. aureus* isolated in the general medical, surgical and orthopaedic wards, and for all strains derived from patients attending the casualty department. In view of the large numbers involved, a representative selection was made for this study by recording the phage types and sensitivity pattern of every third strain isolated from the wounds and sputa of in-patients, and every third strain isolated from wounds in the casualty department. This procedure provided information on 437 strains derived from in-patients and 582 strains isolated from patients attending the casualty department.

At this hospital there is no policy of systematic swabbing of nursing staff for the purpose of detecting nasal staphylococcal carriers. However, during the period under review several hundred nasal swabs from the staff had been examined, for various reasons. In all cases where the growth obtained had consisted predominantly of *Staph. aureus*, the strain had been phage-typed and tested for sensitivity to antibiotics. Such information was available on 57 strains, derived from 52 members of the nursing staff.

RESULTS

Relative incidence of various phage types in the different populations

The incidence of the various phage types in the different populations studied is shown in Table 1. A slight simplification has been introduced in the interests of clarity, namely the grouping together of closely related phage types. For example, the '3c/55/71' group includes strains lysed only by the individual phages 3c, 55 and 71, in addition to those strains which were lysed by all three phages.

Strains of phage type 77 were endemic in the hospital environment, and were apparently almost twice as common among strains isolated within the intensive care unit, when compared with those isolated from patients in general wards. In contrast, this phage type was rarely encountered in patients attending the Casualty department. Strains of phage type 84/85 also appeared to be commoner within the hospital. Unlike strains of type 77, the incidence of type 84/85 strains was lower among staphylococci from the intensive-care unit. The probable explanation lies in the fact that type 77 was distributed throughout the medical and surgical wards of the hospital, whereas type 84/85 was essentially endemic only in the orthopaedic wards (from which comparatively few patients are transferred to the intensive care unit).

Table 1. *The incidence of various phage types among strains of* Staphylococcus aureus *isolated from different groups of hospital patients, and from nursing staff*

	Source of strains			
Phage-type	I.C.U. patients	Other hospital in-patients	Casualty patients	Nursing staff
77	57 (51)*	119 (27)	15 (3)	4 (7)
84/85	6 (5)	39 (9)	4 (1)	1 (2)
'29/52/52A/80 group'	10 (9)	65 (15)	156 (27)	18 (32)
'3C/55/71 group'	5 (5)	19 (4)	121 (21)	9 (16)
42E	1 (1)	6 (1)	33 (6)	1 (2)
Other types	17 (16)	96 (21)	86 (15)	9 (16)
Untypable	15 (14)	93 (21)	167 (29)	15 (26)
Total	111	437	582	57

* Figures in parentheses indicate percentages.

Table 2. *The incidence of resistance to various antibiotics among strains of* Staphyloccus aureus *isolated from the various populations*

	Source of strains			
Resistance pattern	I.C.U. patients	Other hospital in-patients	Casualty patients	Nursing staff
Completely sensitive	5 (5)	63 (14)	219 (36)	16 (28)
Resistant to penicillin only	21 (19)	139 (32)	327 (56)	32 (56)
Resistant to penicillin and two or more others	30 (27)	72 (17)	10 (2)	1 (2)
Methicillin-resistant*	43 (39)	108 (25)	3 (1)	1 (2)
Others	12 (11)	55 (13)	23 (4)	7 (12)
Totals	111	437	582	57

* Methicillin-resistant strains were always resistant to four or five other antibiotics.

Strains belonging to phage type 42E, the '29/52/52A/80' group and the '3c/55/71' group, appeared to be essentially external strains which did not establish themselves permanently to any significant extent within the hospital. The phage types of strains isolated from the noses of the nursing staff corresponded in general with those of the staphylococci obtained from the casualty patients.

Relative incidence of resistance to antibiotics in staphylococci isolated from the different populations

As might be expected, the great majority of strains isolated from casualty patients were either completely sensitive to the antibiotics tested or resistant to penicillin only, while multiple resistance (and methicillin-resistance in particular) were prominent features of the staphylococci derived from in-patients. The

sensitivity-patterns of the staphylococci carried by the nursing staff tended to resemble those of the strains isolated from the lesions of out-patients (see Table 2). Only one of the 52 nurses carried a methicillin-resistant strain in her nose.

Certain correlations existed between phage types and antibiotic sensitivity patterns. For example, 56% of strains in the '29/52/52A/80' group (whether isolated from in- or out-patients) were either completely sensitive or were resistant to penicillin only; strains falling into this group therefore appeared to be endemic in the community and transferred from there into the hospital, where they were responsible for a small number of infections. However, they appeared to lack the ability to develop multiple resistance, and have thus never represented a major proportion of the strains isolated from hospital patients.

Conversely, 85·7% of the 176 strains belonging to phage type 77 which were isolated from hospital in-patients (including intensive-care cases) were found to be multiple-resistant; the majority also displayed methicillin resistance. However, 12 out of the 15 strains of this phage type isolated from casualty patients were either completely sensitive or resistant to penicillin only; the remaining three were multiple-resistant (two being methicillin-resistant), and had almost certainly been acquired through previous exposure to the hospital environment. The probable inference from these findings is that antibiotic-sensitive strains of phage type 77 normally exist as a small proportion of the staphylococci carried by the out-patient community, which, if introduced into the hospital, possess a great capacity for the acquisition of antibiotic-resistance and for spread between patients.

Relation of infection in the intensive care unit to infection in the hospital as a whole

The results presented above superficially suggest that the intensive-care unit has the highest incidence of multiple-resistant, endemic phage-type staphylococci of all the populations studied. To determine whether this was a fair reflexion of the actual position, a more detailed analysis was made of the situations in which the staphylococcal strains from the unit were isolated.

Seventy-six of the 111 strains were isolated from sputum or tracheostomy discharge, and the remainder from non-respiratory sources. Sixty-four of the infections were already present at the time of the patient's admission to the unit (Table 3). Virtually all the staphylococci isolated from patients who had been admitted directly from the community were either sensitive to all antibiotics or resistant to penicillin only. Conversely strains isolated from patients who had been transferred after a stay on another ward showed a high incidence of multiple resistance, and often belonged to phage type 77 or some other phage type essentially characteristic of the hospital environment.

The 47 strains of *Staph. aureus* acquired within the unit were isolated from 45 patients (25 admitted direct, and 20 transferred from other wards). Twenty-two of these strains were isolated from the respiratory tracts of patients in whom some form of artificial airway was operating (endotracheal tube or tracheostomy). Seventeen of these strains belonged to phage-type 77, and 16 showed multiple antibiotic-resistance. The time elapsing between the institution of assisted venti-

Table 3. *Antibiotic resistance in relation to the source from which the patient was admitted to the intensive care unit in* 111 *cases of* Staphylococcus aureus *infection*

Resistance pattern	Infections in patients admitted from outside the hospital		Infections in patients transferred from another ward	
	Infection present on admission	Acquired in I.C.U.	Infection present on admission	Acquired in I.C.U.
Fully sensitive or penicillin-resistant only	13	5	11	3
Multiple-resistant	1	14	39	25

lation and isolation of the staphylococcus from the respiratory tract varied between 1 and 22 days (median 5 days). In 5 of these 22 patients acquiring staphylococcal respiratory tract colonization within the unit there had been a preceding colonization with some other potentially pathogenic species (*Streptococcus pneumoniae* in 2, *Klebsiella* spp. in 3). Antistaphylococcal therapy was followed by colonization with *Pseudomonas aeruginosa* or resistant coliforms in 14 cases.

Of the total of 103 patients with staphylococcal colonizations, 45 were eventually returned to a general ward. On the basis of the specimens submitted to the laboratory, it appeared that the staphylococcal colonization had been eliminated before transfer in all but five of these patients. In three cases the persistent colonization was due to a strain of phage type 77, and it was impossible to assess the exact influence of this common type on the subsequent incidence of staphylococcal infection in the ward. For the remaining two patients the colonizing staphylococcus was of a distinctive phage type, and no further infections due to similar strains were recorded in the transfer wards during the patients' stay.

DISCUSSION

The findings reported here indicate that, in the absence of the selective influence exerted by the hospital environment, most staphylococcal wound and respiratory infections are caused by strains which are either sensitive to all antibiotics or resistant to benzylpenicillin only. However, this applies to less than a third of the staphylococcal colonizations seen in the intensive care unit of this hospital. The chief reason for this is clearly the very high incidence of multiple-resistant staphylococcal infections already present in patients admitted to the unit for respiratory care. Rountree & Beard (1968) found that staphylococci were widely dispersed from such patients, producing extensive contamination of the surroundings, making it difficult to avoid aerial cross-infection of the newly admitted case when patients were nursed in close proximity. The present intensive care unit is far from ideal in this respect, but is probably fairly typical of the accommodation which has been extemporized for this purpose in older hospitals. It is true that most of the isolations, especially those from the respiratory tract, represented colonizations, rather than invasive infection; however, patients nursed in intensive-care units commonly suffer from one or more of the conditions which are known to be

associated with impaired pulmonary bactericidal activity (Wright, 1961; Green & Kass, 1964; Goldstein & Green, 1966) and there is always a possibility of pneumonia if the numbers of colonizing bacteria reach a level sufficient to overwhelm the pulmonary clearance mechanism. Consequently, continual bacteriological monitoring of the respiratory tract is essential, so that clinicians can be apprized of colonization at the earliest possible moment. However, antibacterial therapy given at the stage of asymptomatic colonization is of doubtful value, since it may merely result in the supplanting of a relatively sensitive organism by a more resistant one. Price & Sleigh (1970) showed that it was advantageous to abandon antibiotic therapy in the management of *Klebsiella* colonizations, the sole methods of treatment being tracheal toilet and physiotherapy. More recently, Klastersky, Beuner & Daneau (1971) have shown that these principles can be applied with equal success in the field of methicillin-resistant staphylococcal infections, even when there is radiological evidence of pulmonary consolidation. It has been suggested that methicillin-resistant staphylococci have a special predilection for the respiratory tract (Jensen & Lassen, 1969); however, it is probably more likely that the frequency of their isolation from this site in the intensive care unit merely reflects the endemicity of the organisms in the unit. Certainly a previous study from this laboratory showed no difference in the incidence of respiratory and non-respiratory methicillin-resistant staphylococci in the hospital as a whole (Harris, 1970).

The amount of infection introduced into an intensive-care unit can best be reduced by closer attention to measures for the prevention of infection on the general wards from which the patients are derived. It has been suggested (Stokes *et al.* 1972) that routine screening for the presence of tetracycline-resistant staphylococci in the noses of hospital workers and newly admitted patients is a useful measure in identifying strains which may subsequently become endemic and develop multiple resistance. The findings in the present survey indicate that this policy may not be universally applicable. For example, the great majority of multiple-resistant strains within the hospital belonged to phage type 77, yet 12 out of 15 strains of this phage type isolated from casualty patients were either completely sensitive to antibiotics or resistant to penicillin only. Such strains would remain undetected by the screening method.

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Enrichment procedures for the isolation of *Salmonella*, *Arizona*, *Edwardsiella* and *Shigella* from faeces

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SUMMARY

Strontium selenite A broth incubated at 37° C., and strontium chloride B broth incubated at 43° C., have been found an effective combination for the isolation of *Salmonella*, *Arizona* and *Edwardsiella* from human faeces.

Direct plating on deoxycholate citrate agar was superior to enrichment methods for the isolation of *Shigella* species.

Strontium selenite A broth was suitable for the isolation of *Salmonella* and *Arizona* bacteria at both 37° and 43° C.

Strontium chloride B broth incubated at 43° C. was best for the isolation of *Salmonella* and *Edwardsiella*.

Modified bismuth sulphite agar, although superior to D.C. agar for the growth of *Salmonella* and *Arizona* bacteria, was unsuitable for *Edwardsiella* and *Shigella* species.

A considerable difference was observed in the distribution of *Salmonella*, *Arizona* and *Edwardsiella* serotypes isolated from humans in remote areas, when compared with isolations from more densely populated urban and agricultural centres.

Epidemiological and zoogeographical aspects of host–parasite associations between humans, animals and pathogenic Enterobacteriaceae in Western Australia are discussed.

INTRODUCTION

Strontium hydrogen selenite and strontium chloride have been found effective as selective ingredients in *Salmonella* enrichment media (Iveson & Mackay-Scollay, 1969, 1972). In comparative tests, strontium chloride malachite green broth (strontium chloride M) was found suitable for the isolation of salmonellas from human and animal faeces, foodstuffs, meat processing and abattoir effluents. The medium compared favourably with Rappaport's broth and, except for the isolation of *S. typhi*, was superior to selenite F. Strontium selenite broth was superior to selenite F, tetrathionate, Rappaport and strontium chloride M media for the isolation of *S. typhi*.

Similar results were reported by Chau & Huang (1971), who found strontium selenite broth superior to selenite F for the isolation of *S. typhi*. The method was also suitable for the isolation of the host-specific serotypes, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. abortusequi* and *S. sendai* from the faeces of

hospital patients who were admitted without fever or enteric symptoms. Strontium chloride M and Rappaport were satisfactory for the isolation of *S. choleraesuis* but unsuitable for the recovery of *S. typhi*. Strontium chloride M was also found suitable for the isolation of *Salmonella* from bovine specimens (Miller, 1971).

In further investigations it was shown that the selective qualities of strontium chloride M broth were only slightly reduced when the malachite green dye was omitted, furthermore the modified medium (strontium chloride B broth) was found suitable for the isolation of *Edwardsiella tarda*, as well as *Salmonella* and *Arizona* bacteria (Iveson, 1971). The medium was well suited to the elevated temperature technique, and multiple salmonella serotypes were recovered from abattoir effluents using strontium chloride B broth incubated at 43° C. (Iveson & Mackay-Scollay, 1972).

In the present investigations, comparisons of media have been undertaken to evaluate the performance of strontium selenite A and strontium chloride B enrichment media, for the isolation of *Salmonella*, *Arizona*, *Edwardsiella* and *Shigella* from human faeces. Specimens were collected from both urban and remote areas and the techniques involved and results obtained are presented in this report.

MATERIALS AND METHODS

Specimens

A total of 12,807 faeces specimens were examined from patients with gastro-enteritis or from selected contacts throughout Western Australia. Faeces samples of 2–10 g. were collected into 10 ml. volumes of Sachs (1939) faeces transport medium, and occasionally into dry sterile bottles. Approximately 5000 of the samples were collected in the sparsely populated semi-tropical Kimberley region in the far north of Western Australia, and were transported by air to the central laboratories. The transit period varied from 1 to 3 days.

Culture procedure

Faeces samples were mixed with the transport medium and inoculated direct on Oxoid deoxycholate citrate (D.C.) agar, and approximately 1·0 ml. was added to 10 ml. volumes of the particular enrichment media used in a series of six complementary studies.

The first, study I, involved the testing of 4958 faeces specimens using direct culture on D.C. agar, strontium selenite broth prepared with potassium dihydrogen phosphate, and strontium chloride B enrichment broth incubated at 37° C. Study II was directed particularly to investigate the performance of strontium chloride B broth incubated at 43° C., and a total of 1950 faeces samples were examined by the elevated-temperature technique, as well as by the methods used in the first study.

Study III was undertaken to evaluate the performance of strontium selenite broth prepared with di-sodium hydrogen phosphate (strontium selenite A broth). A total of 5465 faeces specimens were examined using strontium selenite A broth incubated at 37° C. and strontium chloride B broth incubated at 43° C. In study IV

particular attention was directed to assess the performance of strontium selenite A and strontium chloride B, when used in parallel with G.N. broth (B.B.L.).

Studies V and VI involved the repeat testing of selected faeces samples from which Salmonella, Arizona or Edwardsiella species had been previously isolated. The repeat examinations were performed on specimens stored at room temperature for periods of 7–14 days in the laboratory. In study V, 131 positive samples were repeat-tested by direct culture and strontium selenite, strontium chloride B, strontium chloride M and selenite F (Oxoid) incubated at 37° C, and in the final study, 140 positive specimens were repeat-tested using strontium selenite A, strontium chloride B and Rappaport enrichment media incubated at 37° and 43° C.

Media

Strontium selenite A enrichment broth

Bacto tryptone (Difco)	0·5 g
Sodium chloride	0·8 g
Di-sodium hydrogen phosphate (Na_2HPO_4)	0·05 g
Strontium hydrogen selenite (Ajax)	0·2 g
Distilled water	100 ml

The strontium hydrogen selenite, $Sr(HSeO_3)_2$, was dissolved in water without heating, the remaining ingredients were added, and the medium distributed in 10 ml. volumes and sterilized by steaming for 30 min. The pH was 6·8 and did not require adjustment. The medium remained stable during storage at room temperature for periods up to 6 months.

Other enrichment broth media

The strontium chloride B, Rappaport and selenite F media were prepared as reported by Iveson (1971). The strontium selenite and strontium chloride M broths were prepared as reported by Iveson & Mackay-Scollay (1972). Gram-negative broth (B.B.L.) was prepared as recommended by the manufacturer.

Subcultures from the enrichment broths were performed after 18–24 hr incubation, on D.C. agar (Oxoid) and modified bismuth sulphite agar (Iveson, 1971). An average of three suspect colonies were selected from each of the plating media, and examined biochemically and serologically.

RESULTS

Salmonella organisms were recovered from 1042 faeces samples, *Arizona* from 11, *Edwardsiella* from 40 and *Shigella* from 738 specimens.

Direct culture procedures recovered 315 (30%) *Salmonella*, 1 (9%) *Arizona*, 9 (23%) *Edwardsiella* and 702 (95%) *Shigella*, whereas enrichment methods recovered 1042 (100%) *Salmonella*, 11 (100%) *Arizona*, 37 (93%) *Edwardsiella* and 139 (18%) *Shigella*. The relative efficiency of the direct and enrichment culture methods used are detailed in Table 1.

Table 1. *Relative efficiency of strontium selenite and strontium chloride* B *Enrichment in four experiments in the isolation of* Salmonella, Arizona, Edwardsiella *and* Shigella *from* 12,807 *faeces samples*

Expt. and no. of samples	Culture method	Temp. (C.)	Species and isolations			
			Salmonella	*Arizona*	*Edwardsiella*	*Shigella*
I (4958)	Direct	37°	121	1	6	393
	Strontium chloride B	37°	267 (334)	1 (1)	20 (21)	52 (406)
	Strontium selenite	37°	289	1	0	21
II (1950)	Direct	37°	68	0	0	75
	Strontium chloride B	37°	197	0	7	7
	Strontium selenite	37°	198 (287)	0 (0)	0 (7)	0 (76)
	Strontium chloride B	43°	257	0	6	3
III (5465)	Direct	37°	112	0	3	212
	Strontium selenite A	37°	307 (379)	10 (10)	0 (9)	53 (229)
	Strontium chloride B	43°	324	1	7	15
IV (434)	Direct	37°	14	0	0	22
	Strontium selenite A	37°	37	0	0	10
	G.N. Broth	37°	18 (42)	0 (0)	0 (3)	6 (27)
	Strontium chloride B	43°	37	0	3	3
Totals (12,807)			(1042)	(11)	(40)	(738)

Table 2. *Relative efficiency of strontium selenite, strontium chloride, Rappaport and selenite* F *for the isolation of* Salmonella, Arizona *and* Edwardsiella *from* 271 *faeces specimens*

Expt.	Samples	Culture method	Temp. (C.)	Species and isolations		
				Salmonella	*Arizona*	*Edwardsiella*
V	131	Direct	37°	30	0	3
		Strontium selenite		80	1	0
		Strontium chloride B		70 (100)	0 (1)	13 (13)
		Strontium chloride M		70	0	0
		Selenite F		55	0	0
VI	140	Direct	37°	26	0	2
		Strontium selenite A		90	4	0
		Strontium chloride B		76	0	5
		Rappaport		91 (113)	2 (7)	0 (6)
		Strontium selenite A	43°	98	6	0
		Strontium chloride B		95	1	5
		Rappaport		77	0	0
Totals	271			(213)	(8)	(19)

Salmonellas

In Expts. I and II there was no significant difference in isolations by strontium chloride B and strontium selenite broths incubated at 37° C. In Expt. II the isolations obtained by strontium chloride B incubated at 43° C. were significantly greater than those by either strontium chloride B or strontium selenite incubated at 37° C.

In Expts. III and IV there were no significant differences in isolations by strontium selenite A incubated at 37° C. and strontium chloride B broth incubated at 43° C.

Arizonas

Only 11 strains were isolated, and of these 10 were recovered in Expt. III during a single outbreak of infection associated with Arizona 26:26–25. Strontium chloride B isolated only 1 strain, whilst strontium selenite A incubated at 37° C. isolated 10 strains.

Edwardsiellas

A total of 40 isolations were obtained – 9 by direct plating and 37 by enrichment in strontium chloride B broth incubated at 37° or 43° C. There were no Edwardsiella isolations by any other enrichment broth.

Shigellas

In the four experiments direct culture on D.C. agar was greatly superior to enrichment methods. In a total of 738 Shigella isolations, 599 were recovered by direct culture alone, and 103 by both direct and enrichment culture. A total of 36 (4·9%) isolations were recovered exclusively by enrichment methods. In Expts. I and II the strontium selenite and strontium chloride B enrichment methods increased the direct culture isolation's total from 468 to 482 (3%), compared with an increase of 234–254 (8·5%) by the strontium selenite A broth used in Expts. III and IV. In Expt. IV two Shigella isolations were recorded exclusively from G.N. broth and three from strontium selenite A broth.

Multiple *Salmonella* infections were detected in 71 (7%) positive specimens – 13 samples yielded 3 serotypes, and 58 samples 2 serotypes, on a single examination. *Salmonella* and *Shigella* were recovered together from 22 samples, *Salmonella* and *Edwardsiella* from 9, *Salmonella* and *Arizona* from 5, and *Shigella* and *Edwardsiella* species from 2 samples. A total of 5 specimens also yielded 2 *Shigella* serotypes.

Most of the mixed *Salmonella* infections, and all infections by *Arizona* and *Edwardsiella*, were diagnosed in humans living in remote areas. The geographical distribution and relative frequency of *Salmonella*, *Arizona*, *Edwardsiella* and *Shigella* isolations recovered from the more densely populated urban and agricultural centres located south of the 28° latitude, and in the remote sparsely populated northern regions of Western Australia, are shown in Table 3.

Table 3. *Distribution and relative frequency of* Salmonella, Arizona, Edwardsiella *and* Shigella *isolations in southern and remote northern areas of Western Australia*

Serotype	Isolations from Southern areas	Isolations from Northern areas	Serotype	Isolations from Southern areas	Isolations from Northern areas
S. abony	0	12	*S. paratyphi* A	1	0
S. adelaide	14	28	*S. paratyphi* B	6	1
S. anatum	11	8	*S. poona*	1	4
S. bahrenfeld	0	36	*S. potsdam*	3	0
S. ball	0	1	*S. ramat-gan*	0	2
S. bleadon	0	2	*S. rubislaw*	0	15
S. bovismorbificans	46	6	*S. saintpaul*	6	10
S. bredeney	1	3	*S. senftenberg*	0	16
S. brisbane	0	1	*S. singapore*	2	0
S. bukavu	0	3	*S. taksony*	0	1
S. charity	0	3	*S. tennessee*	2	32
S. chester	33	44	*S. treforest*	0	3
S. derby	38	7	*S. typhi*	2	0
S. eastbourne	0	11	*S. typhimurium*	266	44
S. emmastad	0	6	*S. urbana*	0	3
S. enteritidis	3	1	*S. wandsbek*	0	7
S. fremantle	0	10	*S. wandsworth*	0	37
S. gaminara	0	1	*S. welikade*	0	5
S. give	10	6	*S. zehlendorf*	0	1
S. havana	27	13	*S. species*	0	8
S. hvittingfoss	0	23	A. 9a9b:29:31	0	1
S. jangwani	0	14	A. 26:24:25	0	1
S. java	0	3	A. 26:26:25	0	9
S. kisarawe	0	1	*E. tarda*	0	40
S. kottbus	0	1	*Sh. flexneri* I	27	4
S. lansing	0	3	*Sh. flexneri* II	22	10
S. lexington	2	0	*Sh. flexneri* III	21	0
S. litchfield	0	5	*Sh. flexneri* IV	126	167
S. livingstone	2	1	*Sh. flexneri* VI	101	136
S. manila	0	1	*Sh. flexneri* X	3	1
S. muenchen	57	77	*Sh. flexneri* Y	2	22
S. newbrunswick	2	5	*Sh. boydii* IV	5	9
S. newington	1	1	*Sh. schmitzii*	6	5
S. ohlstedt	0	3	*Sh. sonnei*	54	22
S. oranienburg	13	32	Totals	922	998
S. orientalis	2	0			
S. orion	4	10			

The results of the repeat tests performed on samples found positive during routine testing are detailed in Table 2. In Expt. V, strontium selenite broth prepared with potassium dihydrogen phosphate was superior to strontium chloride B, strontium chloride M and selenite F, for the isolation of *Salmonella* when samples were incubated at 37° C. The three strontium media were each considerably better than selenite F.

In Expt. VI strontium selenite A and Rappaport broth were both superior to strontium chloride B medium for the recovery of *Salmonella* when enrichment media were incubated at 37° C., but in tests at 43° C. strontium selenite A and

strontium chloride B were considerably better than Rappaport's medium. Strontium selenite A was best for the recovery of *Arizona* species.

Strontium chloride B was the only enrichment procedure to recover *Edwardsiella* in the repeat tests, and was superior to direct culture when samples were incubated at 37° and 43° C.

DISCUSSION

Salmonella enrichment media have been designed with the aim of modifying a selected portion of a microbial-ecosystem and achieving, at least for a limited period of time, a cultural schema suitable for the rapid multiplication of the desired bacterial species. At the same time, during the incubation period, undesired competitors must be eliminated or their growth suppressed.

The problems of achieving an efficient single enrichment or plating medium for the isolation of *Salmonella*, *Arizona*, *Edwardsiella* and *Shigella* organisms, has been complicated by the cultural diversity of the desired species, the close association of the culturally similar non-pathogenic Enterobacteriaceae and, in mixed infections, by competition between the desired species which has reduced the chances of detecting the full range of pathogens.

An early insight into the problems of isolating different varieties of bacteria was revealed by Beijerinck (1901), who observed that under the selective conditions of a single enrichment process, it was impossible in most experiments to achieve a relative increase in one species without leading to a disappearance of other organisms. It was also observed that different bacterial species were dominant at different stages of the enrichment culture, however, it was noted that the method made it possible to isolate a large variety of bacteria that were adapted to different environmental conditions.

Salmonella bacteria have been isolated from a wide range of vertebrate hosts, foodstuffs, and contaminated environments, and in an ever increasing species approximately 1600 serotypes have been identified. It has been remarkable in a species of such diversity that only a few serotypes have failed to grow in selenite F or tetrathionate enrichment media, which were introduced primarily to facilitate the isolation of *S. typhi*. Significantly, it has been those serotypes infecting a restricted host range, e.g. *S. typhi*, *S. paratyphi A*, *S. choleraesuis* and *S. abortus-ovis*, that have proved difficult to isolate. On the other hand, if the selective cultural requirements of an important host-specific strain have been met, it has usually followed that the serotypes capable of infecting a wider host range have also grown satisfactorily.

Selenite F broth has been widely accepted as a reliable medium for the isolation of *S. typhi* and the majority of food poisoning serotypes from humans and animals. However, both selenite F and tetrathionate enrichment media have been frequently modified in attempts to improve their performance, and in a recent comprehensive study of the bacterial aetiology of human diarrhoea (Sakasaki, Tamura, Prescott & Bencic, 1971) Rappaport's medium, G.N. broth, and selenite cystine enrichment, together with direct culture on S.S. agar, were preferred for the isolation of *Salmonella*, *Arizona* and *Edwardsiella*.

With the exception of *S. typhi*, Rappaport broth has been superior to both selenite F and tetrathionate for the isolation of *Salmonella* (Collard & Unwin, 1958; Iveson, Kovacs & Laurie, 1964; Hooper & Jenkins, 1965; Iveson & Kovacs, 1967; Iveson & Mackay-Scollay, 1969, 1972). However, the method was found unsuitable for the isolation of *S. dublin* and *S. pullorum* from animals and was not suited to the elevated temperature of incubation (Harvey & Price, 1968).

Enrichment culture methods combined with subculture to solid plating media have been superior to direct-plate cultures for the isolation of the majority of *Salmonella* serotypes. The increase achieved by the selenite F and tetrathionate combination has varied from 33 % (Cook, Frisby & Jebb, 1951) to 164 % (Galton & Quan, 1944). The selenite F and Rappaport combination has increased the direct culture isolations 144 % (Hooper & Jenkins, 1965). Salmonella isolations were increased from 238 to 583 (145 %) by Iveson & Kovacs (1967), who used Rappaport, selenite F and tetrathionate enrichment, and from 34 to 108 (218 %) by Chau & Huang (1971), who used strontium selenite, strontium chloride M, Rappaport and selenite F enrichment, as well as direct plating on S.S. agar.

In the present investigation enrichment methods increased the *Salmonella* isolations obtained by direct plating from 315 to 1042 (231 %), *Arizona* from 1 to 11 (1000 %), *Edwardsiella* from 9 to 40 (344 %), and *Shigella* from 702 to 738 (5 %).

The combination of strontium selenite A broth incubated at 37° C. and strontium chloride B enrichment broth incubated at 43° C. increased *Salmonella* isolations from 112 obtained by direct plating to 379 (238 %), *Arizona* isolations were increased from 0 to 10, *Edwardsiella* 3 to 9 (200 %) and *Shigella* from 212 to 229 (8 %). The new combination was also superior to G.N. broth for the recovery of *Salmonella*, *Edwardsiella* and *Shigella*.

Salmonella infections with multiple serotypes were detected in single samples submitted from patients in both urban and remote areas. A total of 42 (59 %) samples from remote areas yielded more than one serotype, compared with 29 (41 %) from urban centres.

S. typhimurium was the most common serotype recovered from humans in urban areas, but there was a strong bias against the distribution of the organism in mixed infections. For example, *S. typhimurium* with 310 (28 %) isolations was recovered along with other serotypes in only 17 (24 %) mixed infections, whereas *S. derby* with only 45 (3·5 %) total isolations was associated with multiple infections on 18 (25·3 %) occasions. A high frequency of *S. derby* in mixed infections has also been observed by Cherubin & Winter (1970), who suggested that *S. typhimurium* was more frequently involved in person to person transmission, while other serotypes, including *S. derby*, were directly or indirectly foodborne. In the present study, *S. typhimurium* occurred sporadically throughout the 2-year period, and was not directly associated with infected foodstuffs. On the other hand, infected meat products and food-handlers were directly implicated in an outbreak of *S. havana* infections, and *S. derby* was recovered in four of the six mixed infections diagnosed during the outbreak. The two serotypes were also frequently detected in effluent samples from the abattoir supplying the meat centre.

It was also observed that there was a marked difference in the geographical

distribution of serotypes. In the more densely populated and agricultural areas south of the 28° latitude a total of 551 *Salmonella* isolations and 26 serotypes were identified, and of these *S. typhimurium* with 266 (48 %) isolations was the predominant serotype. By contrast, in a total of 571 isolations and 50 serotypes from remote northern areas, only 44 (8 %) *S. typhimurium* isolations were recorded.

A total of 50 (89 %) *Salmonella* serotypes were identified from remote areas, furthermore 30 (54 %) *Salmonella* serotypes and all the Arizonas and Edwardsiellas were isolated exclusively in these areas, a remarkable distribution for a sparsely inhabited region almost 600,000 square miles in area and containing only 50,000 (5 %) of the state's population. There was also a close relationship between the *Salmonella*, *Arizona* and *Edwardsiella* serotypes isolated from Aboriginal patients in remote areas, and strains that have been recovered from reptiles in Western Australia (Iveson, Mackay-Scollay & Bamford, 1969; Iveson, 1971; Iveson & Mackay-Scollay 1972). Surprisingly, 25 of the *Salmonella* serotypes isolated from humans in remote areas have not been detected in humans or domesticated animals in the more closely settled urban and agricultural centres located in southern areas of the state. Differences in the distribution patterns of *Salmonella* and *Arizona* serotypes recovered from reptiles, when compared with isolations from foodstuffs, humans and sewage from nearby urban areas, have been reported by Zwart, Poelma & Strik (1970), who commented that reptiles carry their own pattern of *Salmonella* and *Arizona*, characterized by an impressive spread over a great variety of serotypes.

The distribution pattern of *Salmonella* serotypes identified in the present investigation has suggested that differences between the serotypes occurring in humans and reptiles are less marked in geographically isolated regions inhabited by indigenous human and animal populations, particularly in areas where the effects of agriculture are minimal. Intensive agriculture, including irrigation processes and large-scale mining centres, have only recently been established in the northern Kimberley and Pilbara regions of Western Australia.

The relative absence of *S. typhimurium*, together with increased isolations of salmonellas classified serologically in numerically higher somatic groups, and strains of subgenus II, III and IV in remote areas has been observed previously in Australia (Iveson *et al.* 1969). It was also reported that the geographical distribution of serotypes suggested that many species were established in Australia before the invasion of the region by European man, his introduced fauna and parasites.

Arizona infections were diagnosed mainly in young Aboriginal children, but on one occasion *Arizona* 26:26:25 was isolated from a nurse who had attended an infected child. The predominant symptoms have been acute gastro-enteritis with or without fever, and in several cases admission to hospital has been necessary to effect treatment. Arizona 26:26:25 and Arizona 26:24:25 have also been isolated from humans, reptiles or animal products from the Indian subcontinent (Bhat, Shanthakumari & Myers, 1969; Harvey & Price, 1962; Kaura *et al.* 1971; Sharma, Kaura & Singh, 1970).

Edwardsiellas were isolated from both children and adults with symptoms of gastro-enteritis and occasionally from healthy individuals. From 29 patients

edwardsiellas alone were isolated, whereas from 9 patients salmonellas were also isolated and from 2 shigellas. A total of 11 patients were infected, in addition to *Edwardsiella*, with *Giardia lamblia*, *Ancylostoma duodenale*, *Strongyloides stercoralis* or *Hymenolepis nana*.

Of the 40 infections 34 (85%) were diagnosed in Aboriginal patients and 8 infections occurred in hospital visitors or their children.

The *Edwardsiella* genus has shown considerable serological diversity and, in the 2 years since strontium chloride B broth was introduced 50 serotypes, including 10 serotypes isolated from humans in the present study, have been identified so far, in a total exceeding 500 isolations in Western Australia.

The mechanisms underlying the selective action of strontium hydrogen selenite and strontium chloride, have not been fully investigated. However, strontium selenite A broth has shown a pattern of selenite reduction similar to selenite F. The reduction of selenite to selenium has been slightly less intense, but the characteristic brick-red colour associated with bacterial growth was clearly evident after 16–24 hr. incubation. A concentration of 0·2% strontium hydrogen selenite and 0·05% di-sodium hydrogen phosphate has been found optimum for the recovery of *Salmonella* and *Shigella*, compared with 0·4% sodium hydrogen selenite as used in selenite F. It was found unnecessary to incorporate a fermentable carbohydrate. Both media contained Bacto tryptone and isotonic sodium chloride.

Strontium selenite broth was first prepared using potassium dihydrogen phosphate (Iveson & Mackay-Scollay, 1969), and the pH was adjusted to 6·8 with sodium hydroxide. However, it was observed that improved results were obtained with the strontium selenite A modification, furthermore, at the reduced concentration of 0·05% di-sodium hydrogen phosphate, the optimum pH was achieved without further adjustment.

The ability of certain chloride salts to favour selectively the growth of certain salmonellas was first reported by Gray (1931), who used lithium chloride added to peptone water, and Rappaport, Konforti & Navon (1956), who used a combination of magnesium chloride and malachite green. The dye was added to increase the selectivity of the medium but, although suppressing the growth of undesired competitors, the dye rendered the medium unsuitable for the isolation of *S. typhi*. It was found essential to use Bacto tryptone in order to recover the more fastidious *S. paratyphi* A. Bacto tryptone has been used continuously in the strontium chloride B medium, and has been found particularly effective in promoting the growth of *Salmonella* and *Edwardsiella*. The pH 5·3 combined with the 3·6% strontium chloride concentration has also efficiently suppressed coliforms and *Proteus* species, and the selective action was further improved when samples were incubated at 43° C.

Strontium selenite A and strontium chloride B enrichment media have replaced the enrichment procedures previously used in our laboratories, and have been found an improved combination for the isolation of *Salmonella*, *Arizona*, *Edwardsiella* and *Shigella* from humans, animals and environmental samples. The media have efficiently suppressed the non-pathogenic Enterobacteriaceae and have responded favourably to the elevated temperature of incubation. It has been

found unnecessary to add carbohydrates, accessory growth factors, dyes or antibiotics, and both media have been stored ready for use at room temperature for several months without adverse effects.

Throughout the investigation, strontium chloride B broth incubated at 37° or 43° C. and subcultured to D.C. agar was found best for the isolation of *Edwardsiella* species. Occasionally, however, strains isolated by direct plating were not isolated after enrichment.

Strontium selenite A broth incubated at 37° or 43° C. and subcultured to modified B.S. agar was best for the isolation of *Arizona* species.

Modified B.S. agar was greatly superior to D.C. agar for the isolation of *Salmonella* and *Arizona* species. *Edwardsiella* and *Shigella* species were, however, isolated exclusively on D.C. agar.

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The occurrence of salmonellas and lactose-negative Arizonas in reptiles in The Netherlands, and a comparison of three enrichment methods used in their isolation

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SUMMARY

A survey was conducted in 1971 in healthy reptiles supplied to the Central Animal Laboratory of Nijmegen for experimental animal research. In order to determine which salmonella serotypes occur, and whether there are several serotypes per animal, several strains of each positive sample were typed.

It was found that 160 of 169 samples contained salmonellas or lactose-negative Arizonas or both, and 95 different serotypes were isolated.

Of 127 animals examined individually, 67 were carriers of more than one serotype, 42 animals having two types, 21 three types and 4 animals four types.

Three enrichment methods were compared. These were tetrathionate broth incubated at 37° C. (T37) and at 43° C. (T43), and selenite broth incubated at 37° C. (SB). All were incubated for 48 hr. before subculture on brilliant-green agar plates. The enrichment methods T37, T43 and SB produced 99, 125 and 123 positive samples respectively, when taken separately. The combinations of T37 and T43, T37 and SB, and T43 and SB produced 145, 142 and 150 positive samples respectively.

The yield of serotypes in comparable samples showed no difference between the three enrichment methods. With the use of two methods the yield increased by about 38% compared with one method, and the combination of three methods showed an increase in serotype yield of about 64% compared with one method. A distinct preference by serotypes for definite enrichment methods was not proved.

INTRODUCTION

Amongst reptiles, salmonella carriers are found frequently, but the percentage incidences reported in the literature are rather divergent. Hinshaw & McNeil (1947) found salmonellas in 3 of 12 lizards (25%), Lee & Mackerras (1955) in 35 of 52 lizards (67%), Mackey (1955) in 144 of 301 lizards (48%), Bövre & Sandbu (1959) in 27 of 33 tortoises (82%), de Hamel & McInnes (1971) in 28 of 492 lizards (5·7%), whereas Milanov, Chilev, Pashev & Slavkov (1966) found a salmonella carrier rate of 50–100% in tortoises according to their area of origin. In the Netherlands Zwart (1960) and Zwart, Poelma & Strik (1970) found a large number of

types of salmonellas and Arizonas in reptiles which died at zoological gardens. In an earlier survey (Koopman & Janssen, 1972*b*) we found salmonellas in 5 of 10 reptiles.

In order to trace on a larger scale how many healthy reptiles are carriers, and which serotypes they carry, we examined the reptiles supplied to the Central Animal Laboratory of Nijmegen during one year for salmonellas and lactose-negative Arizonas. We also ascertained whether more than one serotype per animal could be demonstrated.

According to Nitzschke (1951), Leistner, Deibel, Johantges & Niven (1962), Mueller (1963) and Luethgen & Lucas (1971) some serotypes sometimes prefer definite enrichment methods. It seemed to us appropriate to check whether such preference could be established for the methods which we used.

MATERIALS AND METHODS

The reptiles examined were supplied to the Laboratory in 1971 on behalf of experimental animal research. One lizard caught in the Netherlands was examined; the rest of the animals were acquired through trade channels and were not of Dutch origin.

A total of 127 animals were examined individually. In addition 42 pooled samples were examined, comprising 12 samples from 2 animals, 3 samples from 3 animals, 14 samples from 4 animals, 12 samples from 5 animals and one sample from 20 animals.

Shortly after arrival of the animals faeces samples were collected. For this, the animals were kept on filter paper. When faeces had been passed the filter paper with faeces was cut out for examination.

The samples were examined for *Salmonella* by the methods described previously (Koopman & Janssen, 1972*a*), but the size of the sample was often much smaller, and varied from 0·1 to 1 g. Each sample was examined by three different enrichment methods; tetrathionate broth at 37° C. (T 37) and at 43° C. (T 43) and selenite broth at 37° C. (SB). After 48 hr. incubation each tube was streaked on two brilliant-green agar plates (BG plates). Suspicious colonies were subcultured to obtain pure cultures (without biochemical examination being done) and sent to the Laboratory for Zoonoses of the National Institute of Public Health, Utrecht, for typing. Usually several strains from BG plates from positive enrichment media were typed.

RESULTS

Of the 169 faecal samples examined, 160 were positive for salmonellas or lactose-negative Arizonas, or both. Of the 127 animals examined individually 119 (93·7 %) were positive. Of the 42 pooled samples, only one sample was negative.

Table 1 shows the results for each species of reptile, and in Table 2 is shown a list of serotypes isolated, together with their animal hosts. Among the 95 serotypes there were 21 which were isolated for the first time in the Netherlands, and one of these types had not been previously isolated (Salm. 30:c, z39 N-group). Table 3 shows the distribution of multiple-serotype isolations among the different samples.

Table 1. *The number of cultures typed and the number of different types of salmonellas and lactose-negative Arizonas per species of reptile*

Species	No. of animals	No. of samples	No. of positive samples	No. of typed cultures	No. of serotypes
Agama stellio	2	2	2	12	2
Basiliscus basiliscus	2	2	2	10	3
Caiman sclerops	30	10	10	36	9
Chamaeleo jacksoni	1	1	1	3	3
Iguana iguana	4	4	4	20	5
Lacerta agilis	1	1	1	3	1
L. galloti	107	34	34	158	29
L. viridis	52	25	23	98	21
Scincus scincus	5	4	4	19	5
Testudo graeca	1	1	1	6	1
T. hermanni	37	37	34	132	31
Tupinambis nigropunctatis	1	1	1	8	2
T. teguixin	53	47	43	190	26
	296	169	160	695	

For a comparison of the three enrichment methods and their possible combinations use was made of the results from 104 comparable positive samples. From a part of these samples two strains per positive enrichment method were typed. Furthermore, these samples include those from which more than two strains were typed, provided that they resulted in the same serotypes. The result of a negative enrichment is denoted by 0; 1 means that the two strains isolated were of the same serotype, and 2 means that they were of different serotypes.

The number of samples from which salmonellas or Arizonas were isolated through one, two or three enrichment methods is shown in column 2 of Table 4. Columns 3 and 4 show the number of strains examined and the number of serotypes found by each method or combination of methods.

Columns 5 and 6 in Table 4 show the number of samples from which two strains per enrichment medium were typed, and the number of different serotypes found in these. Column 7 shows how many different serotypes per method were obtained from 104 comparable positive samples.

Table 5 shows the number of times 0, 1 and 2 serotypes per enrichment method were found from these 104 samples.

Table 6 gives a list of the serotypes isolated, showing the frequency of the number of samples from which these serotypes were obtained through each enrichment method separately.

DISCUSSION

It appears from the results that about 95 % of the reptiles examined individually were established carriers of salmonellas or lactose-negative Arizonas. In the literature such high percentages have been reported for tortoises only. Our high incidence for other species of reptiles is probably partly due to the methods used;

Table 2. *Salmonella types isolated from reptiles in* 1971

S. abony	*Testudo hermanni*
S. abony var. *haifa*	*Testudo hermanni*
S. abaetetuba	*Lacerta viridis*
	Testudo hermanni
	Tupinambis teguixin
S. alachua	*Lacerta galloti*
*S. alger**	*Testudo hermanni*
S. anatum	*Caiman sclerops*
	Tupinambis teguixin
*S. anecho**	*Basiliscus basiliscus*
	Lacerta galloti
	Iguana iguana
*S. argentina**	*Lacerta galloti*
S. baildon	*Agama stellio*
S. ball	*Tupinambis teguixin*
S. bardo	*Lacerta galloti*
	Tupinambis teguixin
	Testudo hermanni
*S. bellville**	*Lacerta viridis*
S. bilthoven	*L. galloti*
	Testudo hermanni
	Tupinambis teguixin
S. bleadon	*Testudo hermanni*
S. canastel	*T. hermanni*
S. carrau	*Lacerta galloti*
S. cerro	*T. hermanni*
S. cubana	*Scincus scincus*
*S. detroit**	*Lacerta viridis*
*S. florida**	*L. galloti*
S. galiema	*Scincus scincus*
S. gaminara	*Lacerta galloti*
	L. viridis
	Tupinambis teguixin
S. give	*T. teguixin*
S. greenside	*L. galloti*
S. halle	*Testudo hermanni*
	Tupinambis teguixin
S. havana	*Chamaeleo jacksoni*
	Lacerta viridis
	Testudo hermanni
	Tupinambis teguixin
S. heidelberg	*Lacerta viridis*
*S. hillbrow**	*L. galloti*
S. houten	*Tupinambis teguixin*
S. hvittingfoss	*Scincus scincus*
	Testudo hermanni
	Tupinambis teguixin
S. infantis	*Caiman sclerops*
S. jodhpur	*Testudo hermanni*
S. johannesburg	*Caiman sclerops*
S. kottbus	*Lacerta galloti*
	L. viridis
	Testudo hermanni
S. langford	*T. hermanni*
S. lindern	*T. hermanni*
*S. luciana**	*Lacerta viridis*
S. marina	*Iguana iguana*
S. midhurst	*Lacerta galloti*
S. muenchen	*Caiman sclerops*
	Lacerta galloti
S. napoli	*L. viridis*
*S. nashua**	*Testudo hermanni*
*S. neasden**	*Lacerta galloti*
S. new-brunswich	*Tupinambis teguixin*
S. newport	*Caiman sclerops*
	Testudo hermanni
	Tupinambis nigropunctatis
	T. teguixin
S. ngozi	*Lacerta galloti*
S. nima	*L. galloti*
	L. viridis
S. oranïenburg	*L. galloti*
	Testudo hermanni
	Tupinambis teguixin
S. pomona	*Basiliscus basiliscus*
	Iguana iguana
	Lacerta galloti
	L. viridis
	Testudo hermanni
S. poona	*Tupinambis teguixin*
S. potsdam	*Testudo graeca*
	T. hermanni
*S. pumila**	*Basiliscus basiliscus*
	Iguana iguana
	Lacerta galloti
	L. viridis
	Testudo hermanni
*S. rhône**	*Lacerta galloti*
S. rubislaw	*Testudo hermanni*
S. san diego	*Tupinambis teguixin*
S. saphra	*Tupinambis teguixin*
*S. sendai**	*Testudo hermanni*
S. sheffield	*T. hermanni*
S. siegburg	*Lacerta galloti*
	L. viridis
S. sladun	*Testudo hermanni*
S. sofia	*T. hermanni*
S. sofia var. 27*	*Lacerta agilis*
*S. souza**	*L. viridis*
S. stanleyville	*Scincus scincus*
*S. suelldorf**	*Tupinambis teguixin*
*S. tosamanga**	*Scincus scincus*
*S. tel-hashomer**	*Agama stellio*
S. tennessee	*Testudo hermanni*
S. typhimurium X ORS	*Caiman sclerops*
S. typhimurium I ORS	*C. sclerops*
	Lacerta galloti
S. typhimurium var. Copenhagen XX 652	*L. viridis*
S. typhimurium var. Copenhagen I ORS	*L. galloti*
S. uphill	*L. galloti*
	L. viridis
	Testudo hermanni

Table 2 (*cont.*)

S. vaertan	*Tupinambis teguixin*	*S.* F-group	*Caiman sclerops*
S. vejle	*L. viridis*	*S.* H-group	*Lacerta galloti*
*S. veneziana**	*L. viridis*	*S.* rough	*L. viridis*
*S. virginia**	*L. galloti*		*Testudo hermanni*
S. warragul	*L. galloti*		*Tupinambis teguixin*
S. wassenaar	*Caiman sclerops*		
S. 30:c, z39 (N-group)*	*Lacerta galloti*	*Ar.* 9abc: 24–34	*Lacerta galloti*
		Ar. 5:13–15	*Iguana iguana*
	Tupinambis nigropunctatis	*Ar.* 24:25–26	*Tupinambis teguixin*
	T. teguixin	*Ar.* 1,4:24–38	*Chamaeleo jacksoni*
S. 58a-sub II	*T. teguixin*	*Ar.* 22:1, 2, 5, 6	*C. jacksoni*
S. 44:z4, z23 sub. II	*Lacerta viridis*	*Ar.* 26:32–31	*Tupinambis teguixin*
S. B-group	*Tupinambis teguixin*	*Ar.* 11:16, 17, 18	*Lacerta viridis*
S. C1-group	*Testudo hermanni*	*Ar.* 26:32–21	*Tupinambis teguixin*

* First isolation in The Netherlands.

Table 3. *The division of the positive samples, according to the number of different types of salmonellas and lactose-negative Arizonas per sample**

No. of samples	No. of positive samples	No. of samples with			
		1 type	2 types	3 types	4 types
127 (1)*	119	52	42	21	4
12 (2)	11	4	5	2	0
3 (3)	3	1	2	0	0
14 (4)	14	4	7	3	0
12 (5)	12	3	4	5	0
1 (20)	1	0	1	0	0
169	160	64	61	31	4

* The number of cultures examined per positive sample varies from 1 to 8. The figures in parentheses correspond with the number of animals per sample.

Table 4. *Results of the investigation of* 169 *samples*

Method of enrichment (1)	No. of positive samples (2)	No. of strains investigated (3)	No. of serotypes isolated (4)	Two strains determined		Two or zero strains determined pro enrichment method of 104 positive samples* (no. of serotypes) (7)
				No. of samples (5)	No. of serotypes (6)	
T37	99 (58, 6%)	187	55	69	44	41
T43	125 (74, 0%)	259	61	99	49	44
SB	123 (72, 8%)	269	61	93	51	43
T37+T43	145 (85, 8%)	446	72			57
T37+SB	142 (84, 0%)	456	80			58
T43+SB	150 (88, 8%)	528	85			61
T37+T43+SB	160 (94, 7%)	715	95			70

* A negative enrichment method yielded 'zero' strains.

Table 5. *The number of times that zero, one and two serotypes were found through three enrichment methods in the investigation of zero or two strains of* 104 *comparable positive samples*

No. of serotypes	T 37	T 43	SB
0*	46	22	27
1	46	70	65
2	12	12	12
No. of samples	104	104	104

* Here the enrichment methods were negative, so the yield of serotypes was 'zero'.

none of the investigators mentioned in the introduction used three enrichment methods. There were many different types (95) in our material, and this has been noted by other investigators of reptiles (Boycott, Taylor & Douglas, 1953; Darasse, Le Minor & Lecomte, 1959; Zwart *et al.* 1970). We isolated a number of lactose-negative Arizonas, whereas they are often lactose-positive (Cowan & Steel, 1965). It is therefore possible that Arizonas occur more frequently than appears from our results.

It is remarkable that the animals were often carriers of several serotypes. More than half the animals examined individually harboured more than one serotype. It might be expected that if more cultures per animal were investigated even more serotypes might be found. Previously, the occurrence of several serotypes per animal has only been recorded for tortoises (Boycott *et al.* 1953; Dimow, 1964).

The source from which our animals are bought is the same as for private persons, so that the extent of contamination in reptiles bought as domestic pets will be as great as in our material. In order to trace whether in the Netherlands a relation exists between the serotypes of *Salmonella* found in men and in our reptiles we have compared the 15 serotypes occurring most frequently in the two groups (Table 7). It is seen that only *S. newport* is common to both groups. A number of the serotypes found commonly in reptiles were not isolated from men in 1971, and conversely. In Dutch conditions the risk of human infection from reptiles seems to be less than might have been expected. It is remarkable that an isolation for the first time in the Netherlands, *S. sofia* Var. 27, was from a reptile caught in this country, *Lacerta agilis*.

To minimize the risk of infection for our staff and for the other animals at our institute the reptiles are kept isolated. Up to now this way of housing has been successful.

It appears from Table 4 (column 2) that T 43 and SB give much better results than T 37. In a previous investigation with dogs and cats it was T 37 which proved to be the most satisfactory (Koopman & Jansen, 1972*a*). This shows that it is best to use various enrichment methods in parallel when one desires one standard method only for different animal species. In comparing the three enrichment methods by the yield of the different serotypes (Table 4, columns 5 and 6) there is little difference between the various media. This is striking because considerably

Table 6. *Serotypes isolated and the frequency with which samples from these serotypes were isolated through the various enrichment methods**

Serotypes	T37	T43	SB	Serotypes	T37	T43	SB
S. abony	0	0	1	*S. poona*	0	0	1
S. abony var. *haifa*	0	0	1	*S. potsdam*	3	1	3
S. abaetetuba	4	3	0	*S. pumila*	7	4	8
S. alachua	1	1	0	*S. rhône*	0	0	2
S. alger	0	1	0	*S. rubislaw*	0	0	1
S. anatum	1	6	1	*S. san diego*	1	0	6
S. anecho	3	1	3	*S. saphra*	7	6	8
S. argentina	3	1	6	*S. sendai*	1	1	1
S. baildon	1	1	2	*S. sheffield*	6	5	3
S. ball	0	0	1	*S. siegburg*	2	0	0
S. bardo	3	1	4	*S. sladun*	1	1	1
S. bellville	0	1	0	*S. sofia*	0	1	1
S. bilthoven	2	4	3	*S. sofia* var. 27	1	1	1
S. bleadon	0	1	0	*S. souza*	1	0	0
S. canastel	0	0	1	*S. stanleyville*	0	1	0
S. carrau	0	2	0	*S. suelldorf*	1	1	1
S. cerro	0	0	1	*S. tosamanga*	1	0	0
S. cubana	1	0	1	*S. tel-hashomer*	1	1	0
S. detroit	0	3	4	*S. tennessee*	1	1	1
S. florida	0	1	0	*S. typhimurium* X ORS	0	0	1
S. galiema	1	1	1	*S. typhimurium* I ORS	1	2	0
S. gaminara	3	4	7	*S. typhimurium* var.			
S. give	0	2	1	Copenh. XX 652	0	0	1
S. greenside	0	0	1	*S. typhimurium* var.			
S. halle	1	4	5	Copenh. I ORS	0	1	0
S. havana	6	6	1	*S. uphill*	0	0	2
S. heidelberg	0	1	0	*S. vaertan*	1	0	0
S. hillbrow	1	0	1	*S. vejle*	0	1	0
S. houten	0	0	2	*S. veneziana*	0	0	1
S. hvittingfoss	2	2	2	*S. virginia*	0	0	1
S. infantis	0	1	0	*S. warragul*	1	0	0
S. jodhpur	0	3	0	*S. wassenaar*	1	1	0
S. johannesburg	0	1	0	*S.* 30:c, z39 (N-group)	2	3	3
S. kottbus	1	6	1	*S.* 58a-sub. II	0	1	0
S. langford	1	0	0	*S.* 44:z4, z23 sub. II	0	0	2
S. lindern	1	0	1	*S.* B-group	0	0	1
S. luciana	1	0	1	*S.* C1-group	1	1	0
S. marina	1	0	1	*S.* F-group	0	1	0
S. midhurst	1	1	1	*S.* H-group	0	1	0
S. muenchen	1	3	1	*S.* rough	3	0	0
S. napoli	1	0	2	*Ar.* 9abc:24–34	0	0	1
S. nashua	1	0	0	*Ar.* 5:13–15	0	1	1
S. neasden	1	2	1	*Ar.* 24:25–26	0	1	4
S. new-brunswich	2	2	1	*Ar.* 1, 4:24–38	0	0	1
S. newport	5	10	3	*Ar.* 22:1, 2, 5, 6	0	0	1
S. ngozi	4	3	1	*Ar.* 26:32–31	1	1	0
S. nima	3	4	0	*Ar.* 11:16, 17, 18	2	2	5
S. oranienbrug	7	10	6	*Ar.* 26:32–21	0	0	2
S. pomona	7	16	11				

* A negative enrichment method yielded 'zero' strains.

Table 7. *Comparison of the* 15 *Salmonella serotypes occurring most frequently in men in The Netherlands and the* 15 *serotypes occurring most frequently in reptiles at the Central Animal Laboratory, Nijmegen, in* 1971

	Salmonella serotypes isolated from men		Salmonella serotypes isolated from reptiles	
1	*typhimurium*	(+)*	*pomona*	(+)†
2	*panama*	(−)	*saphra*	(−)
3	*infantis*	(+)	*oranienburg*	(+)
4	*enteritidis*	(−)	*newport*	(+)
5	*brandenburg*	(−)	*pumila*	(−)
6	*heidelberg*	(+)	*gaminara*	(+)
7	*stanley*	(−)	*havana*	(+)
8	*thompson*	(−)	*halle*	(−)
9	*derby*	(−)	*sheffield*	(−)
10	*agona*	(−)	30:c, z39	(−)
11	*newport*	(+)	*argentina*	(−)
12	*eimsbuettel*	(−)	*bilthoven*	(−)
13	*montevideo*	(−)	*sandiego*	(+)
14	*bredeny*	(−)	*detroit*	(−)
15	*muenchen*	(+)	*bardo*	(−)

* +, Isolated from reptiles; − not isolated from reptiles (1971).
† +, Isolated from men; −, not isolated from men (1971).

fewer T 37 samples were concerned in the comparison than samples of the other enrichment media.

A total of 104 samples could be used for a direct comparison of the number of serotypes found with the three enrichment methods and their combinations. It appears from this that there is little difference between the three methods. The yield increases as more enrichment methods are used in parallel. It is to be expected that the use of more than three enrichment methods would give a further increase in yield, but this increase would probably not counterbalance the additional work and materials.

When, in the case of the 104 samples, we consider how many times an enrichment method is negative, and how many times one or two serotypes were found from the two isolated strains, it appears that T 37 gives the poorest yield, in the sense that this method was the most negative. This was to be expected because this method, in the total results, also showed the lowest yield of positive results.

Although it appears that the affinity for a single type to the three enrichment methods was not equal every time, it was not possible to find a preference of certain serotypes for one of the three enrichment methods.

We have to express our thanks to Mr J. M. de Hulster for the data put at our disposal.

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Dysentery in general practice: a study of cases and their contacts in Enfield and an epidemiological comparison with salmonellosis

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SUMMARY

During a laboratory study of diarrhoea in a large urban area during the years 1953–68, 5319 Sonne dysentery infections in 2620 households and six cases of Flexner dysentery in five households were confirmed. The annual incidence per 10,000 population fluctuated between 2 and 31 with an average of 12.

Shigella sonnei was found in 10 % of new cases of diarrhoea investigated. Plural infections were found in 58 % of the households studied and 30 % of all contacts examined were shown to be infected. Among these contacts the infection rate was higher for children (42 %) than adults (20 %), and higher for adult females (24 %) than males (16 %). The duration of infection was under 2 weeks in nearly half the index cases followed up and longer than 2 months in only 3 %. Intermittent excretion was observed in 15 %. Repeated Sonne dysentery infection and illness was observed after intervals as short as 5 months. Antibiotic sensitivity was recorded after 1955 and colicine typing after 1960. Changes in antibiotic sensitivity were noted. Children of primary-school age were most susceptible to Sonne dysentery, but symptoms in ill cases were as severe in adults as in children. During years of high incidence cases were concentrated within a small number of primary schools.

Sh. sonnei infections were compared with salmonella infections studied concurrently. Sonne dysentery was a winter disease and most cases were under 11 years old, and case-to-case infection was the usual means of spread. Salmonellosis was prevalent in summer and affected nearly as many adults as children, often persons sharing an article of food. It was more prolonged and severe than Sonne dysentery, but less infectious. In all these respects *S. typhimurium* occupied an intermediate place between Sonne dysentery and 'other salmonellas'.

INTRODUCTION

Bacillary dysentery is a common cause of diarrhoea in England and many outbreaks have been well documented. However, its importance in general practice has been studied less than in outbreaks. Such a study has been made over the years 1953–68 in parallel with a study of salmonellosis already reported (Thomas & Mogford, 1970), among a population of approximately 274,000 in general practices in Enfield. The family doctors in the area, the local health department,

and the Edmonton Public Health Laboratory co-operated in the diagnosis and observation of the cases. The findings are reported below.

METHODS

All bacteriologically confirmed dysentery infections were recorded on cards and filed in the laboratory. The first case of dysentery in any household, referred to the laboratory spontaneously by a general practitioner, was listed as an index case. Such cases were defined as *GP index cases* and the home was visited by a Health Inspector. When there were known cases of dysentery in schools or nurseries, samples from suspected contacts were often sent by a medical officer of health to the laboratory for confirmation. Further, the medical officer of health enlisted the co-operation of the school head-teachers to exclude from school any child who developed diarrhoea or vomiting. These children were referred to their family doctors and not re-admitted until a negative bacteriological report had been received. Infections discovered by such means were listed as *MOH index infections* and they and their households were followed up in the same way as GP index cases. Infected households without a determinable index infection, discovered during the follow-up of contacts, were also visited and listed separately. Wherever possible a record was made on the card of the age, sex, school attended or occupation of each member of the household, and also the date of onset of any symptoms. Each person was asked to send a faecal sample to the laboratory. First specimens were usually received before the start of any treatment. Thereafter the patients were treated by their doctors in various ways.

Patients in whose faeces shigellas were found were asked to send weekly specimens, starting three or more days after concluding any antibacterial treatment, until a negative result had been obtained. The rest of the family were then re-examined. Most families were persuaded to remain under observation until two or three consecutive negative specimens had been collected from each infected person. Negative results from unsatisfactory or dry specimens were disregarded.

Ninety per cent of faeces specimens sent for diagnosis from GP index cases were examined during the first week of illness. Microscopy was followed by culture on MacConkey and on deoxycholate citrate agar plates before and after selenite-F enrichment broth. A second specimen was requested from patients with cellular stools, if no pathogen was isolated from the first. Common pathogens were recognized by conventional methods while rarer species were identified at the Dysentery and Salmonella Reference Laboratories. Colicine typing of *Sh. sonnei* strains was done by the technique of Abbott & Graham (1961) and drug sensitivity by standard disk methods.

RESULTS

The estimated total population concerned in the survey at its mid-point was 273,857. Of these, 53,699 were children aged under 15 years.

Table 1. *Dysentery index infections according to year*

Year	*Sh. sonnei* GP index cases	*Sh. sonnei* MOH index infections	*Sh. flexneri*, GP index cases	Total
1953	127	22	—	149
1954	131	38	—	169
1955	43	16	—	59
1956	184	70	—	254
1957	63	8	—	71
1958	16	—	—	16
1959	125	10	1	136
1960	293	109	—	402
1961	33	10	—	43
1962	192	164	—	356
1963	111	32	2	145
1964	85	86	1	172
1965	111	168	—	279
1966	44	2	1	47
1967	114	51	—	165
1968	43	34	—	77
Total	1715	820	5	2540

Incidence of ascertained infection

The annual rate of *Sh. sonnei* infection varied from 2 to 31 per 10,000 population, with a mean of 12. A total of 5319 individual infections were recorded in 2620 households. There were 1715 GP index cases and 820 were MOH index infections. A further 80 infected households with 166 infected children and 56 infected adults were found during the follow up of contacts. Five infected households with 11 infected persons were found during the screening of new nursery entrants. There were also five index cases and one contact with *Shigella flexneri* infections. Thus, there were episodes of infection in 2625 households during the 16 years and 4·4 % of all households in the area are known to have been affected. Most of these households included primary-school children. Among the MOH index infections 47 % of pre-school and 57 % of school age children admitted to symptoms.

Table 1 shows the annual numbers of dysentery index infections and Fig. 1 shows the incidence in each month for all 16 years combined. The greatest numbers of cases were diagnosed in the months January to May.

Dysentery was notified under the provisions of the 1936 Public Health Act. Fig. 2 shows the annual notification rates per 10,000 population in Enfield, in Greater London and in England and Wales. Average annual notification rates in the three areas were 13·0, 7·0 and 6·5 respectively. The Enfield rates seemed to follow a local pattern and were below the others for years of low incidence, but were much higher during local epidemic years. The notified cases from Enfield were bacteriologically confirmed and included index and contact infections. The England and Wales notifications corresponded closely to total dysentery isolations reported by the Public Health Laboratory Service (CMO, 1953–68).

Except where otherwise specified the following observations refer to *Sh. sonnei*.

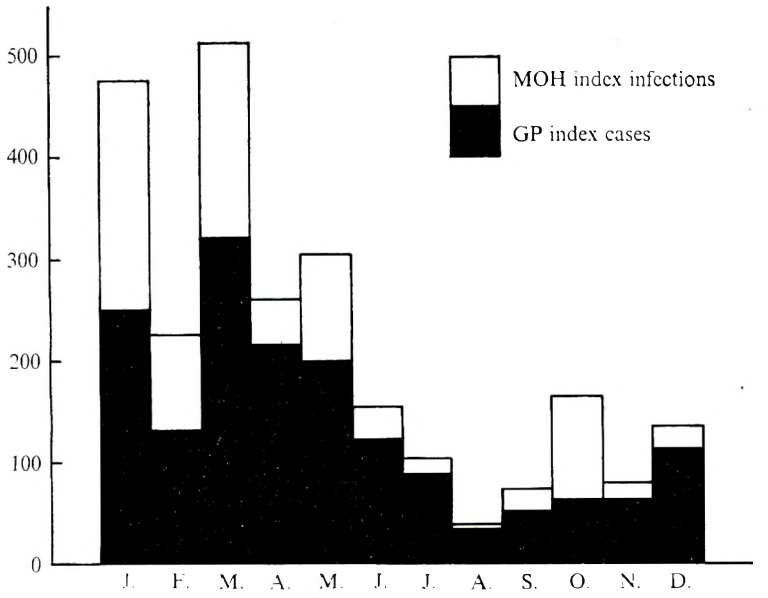

Fig. 1. Incidence of Sonne dysentery according to month.

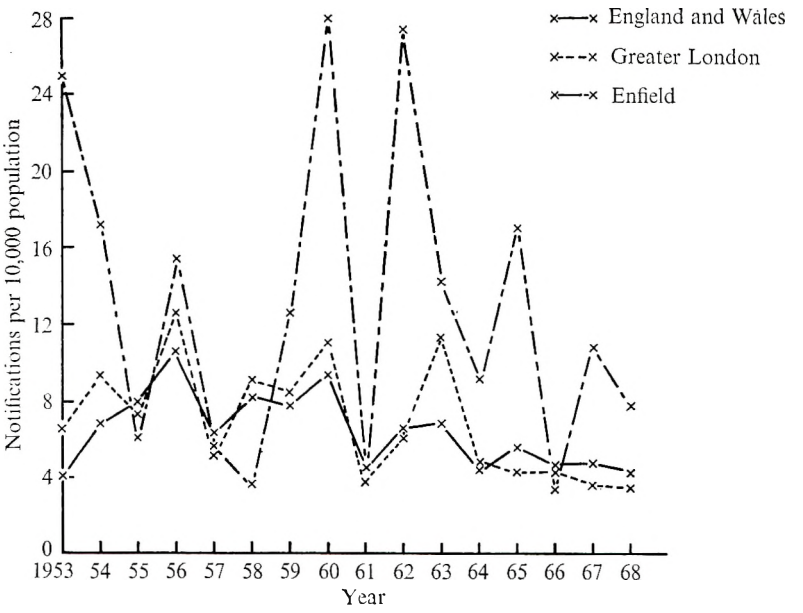

Fig. 2. Annual notification rates to the Registrar General of dysentery in England and Wales, Greater London and Enfield.

Age and sex

Table 2 shows the average annual incidence of index infections per 10,000 of population in different age groups. By far the highest incidence was in the age group 5–9 years, especially of MOH index infections. Within the 10–14 year age group 75 (47%) of the 160 GP index cases and 33 (66%) of the 50 MOH index

Table 2. *Sonne dysentery: incidence according to age group*

Age group	Total no. of GP index cases	Total no. of MOH index infections	Mid-period population	Annual incidence/ 10,000 population of GP index cases	Annual incidence/ 10,000 population of GP index cases and MOH index infections
0–4 years	370	217	17,343	13·3	21·2
5–9 years	783	505	15,676	31·2	51·4
10–14 years	160	50	20,680	4·8	6·3
Child age group unknown	46	5	—	—	—
All children	1359	777	53,699	15·8	24·9
Adult 15+	356	43	220,158	1·0	1·1
Total	1715	820	273,857	3·9	5·8

Table 3. *Sonne dysentery: severity of GP index cases according to age group and sex*

Age and sex group	Admitted early to hospital	Diagnosed at home			Total
		Cells found	No cells found	Not recorded	
Child					
Male	31 (4%)	237 (34%)	427 (61%)	—	695
Female	27 (4%)	285 (43%)	347 (52%)	5 (1%)	664
Adult					
Male	5 (4%)	50 (41%)	65 (53%)	2 (2%)	122
Female	13 (6%)	111 (47%)	106 (45%)	4 (2%)	234
Total	76 (4%)	683 (40%)	945 (55%)	11 (1%)	1715

infections were aged 10 and attended primary schools. Only 102 index infections were found among secondary-school children. Among children there were slightly more male than female index cases – 695 as compared with 664 GP index cases and 399 as compared with 378 MOH index infections. Most of this small male excess was in the 5–9 age group. Among adults there were far fewer male than female index cases – 122 as compared with 234 GP index cases and 6 as compared with 37 MOH index infections.

Severity of illness in GP index cases

These 1715 index cases were sufficiently ill to have called in a doctor. Two criteria were used to assess the severity of index cases – early admission to hospital for medical (not social) reasons, and a record of blood or pus cells in the faeces. Seventy-six (4%) of the 1715 GP index cases were admitted to hospital (Table 3). Among the 1639 (96%) treated at home, cells were found in the first faeces specimen of 683 (40% of 1715), thus 44% were considered to have had a severe illness. Of the 170 faecal samples examined for cells in the second week of illness 29 (17%) were found still to have cells present. A slightly higher proportion of female than of male children had cells in the stools ($P < 0{\cdot}001$). The severity of illness showed no

Table 4. *Antiobiotic resistance of* Sh. sonnei *strains tested*, 1955–1968

	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968
Sulphonamide, 300 μg.														
No. tested	—	26	5	10	106	223	41	181	115	79	107	33	55	1
No. resistant	—	23	5	9	90	134	15	137	101	71	100	29	55	0
Percentage	—	88%	—	—	85%	60%	37%	76%	88%	90%	93%	88%	100%	—
Tetracycline, 50 μg.														
No. tested	—	2	5	6	74	223	41	193	116	79	107	35	54	24
No. resistant	—	1	5	6	53	114	11	15	3	16	44	7	7	2
Percentage	—	—	—	—	72%	51%	27%	8%	3%	20%	41%	20%	13%	8%
Streptomycin, 25 μg.														
No. tested	—	2	1	5	106	148	1	74	116	78	107	35	55	1
No. resistant	—	1	0	3	0	11	0	20	24	15	65	20	51	1
Percentage	—	—	—	—	0%	7%	—	27%	21%	19%	61%	57%	93%	—
Chloramphenicol, 50 μg.														
No. tested	—	20	5	10	105	161	40	191	116	79	107	33	—	—
No. resistant	—	0	0	0	0	0	0	1	0	0	1	0	—	—
Percentage	—	0%	—	—	0%	0%	0%	1%	0%	0%	1%	0%	—	—
Nitrofurazole, 100 μg.														
No. tested	—	—	—	—	—	175	41	180	116	79	106	44	118	70
No. resistant	—	—	—	—	—	9	0	1	0	1	0	0	0	1
Percentage	—	—	—	—	—	5%	0%	1%	0%	1%	0%	0%	0%	1%
Kanamycin, 30 μg.														
No. tested	—	—	—	—	—	—	—	—	—	56	66	49	119	145*
No. resistant	—	—	—	—	—	—	—	—	—	0	0	4	0	43
Percentage	—	—	—	—	—	—	—	—	—	0%	0%	8%	0%	30%
Nalidixic acid, 30 μg.														
No. tested	—	—	—	—	—	—	—	—	—	—	—	23	126	70
No. resistant	—	—	—	—	—	—	—	—	—	—	—	0	2	0
Percentage	—	—	—	—	—	—	—	—	—	—	—	0%	2%	0%
Ampicillin, 25 μg.														
No. tested	—	—	—	—	—	—	—	—	—	—	—	6	59	65
No. resistant	—	—	—	—	—	—	—	—	—	—	—	3	18	7
Percentage	—	—	—	—	—	—	—	—	—	—	—	—	31%	11%

NB. Percentages are only given when 20 or more strains were tested.
* Including strains tested from a school outbreak beginning in 1967.

Table 5. *Sonne dysentery: duration of infection in GP index cases according to age group and number of terminating negative specimens*

Age group	No. of cases	< 2 weeks	2 to < 4 weeks	4 to < 8 weeks	2 months and longer
		Three or more negatives			
0–4 years	200	93 (47%)	64 (32%)	31 (16%)	12 (6%)
5–14 years	496	194 (39%)	167 (34%)	124 (25%)	11 (2%)
< 15 [age unknown]	15	9	3	3	—
15+	159	110 (69%)	32 (20%)	16 (10%)	1 (1%)
Total	870	406 (47%)	266 (31%)	174 (20%)	24 (3%)
		Two negatives only			
0–4 years	104	47 (45%)	32 (31%)	20 (19%)	5 (5%)
5–14 years	342	124 (36%)	127 (37%)	80 (23%)	11 (3%)
< 15 [age unknown]	11	7	3	1	—
15+	108	73 (68%)	23 (21%)	11 (10%)	1 (1%)
Total	565	251 (44%)	185 (33%)	112 (20%)	17 (3%)

relationship to the colicine type or drug sensitivity of prevailing strains of *Sh. sonnei*. Although the incidence of dysentery was very much greater in children it was noted that among ill index cases symptoms were of broadly similar severity in children and adults. No deaths were recorded.

Drug sensitivity and treatment

Samples of strains isolated from cases and excreters were tested for drug resistance from 1956 onwards. Table 4 shows results of sensitivity tests to the eight most commonly used antibacterial drugs. The rate of sulphonamide resistance was high, but fell temporarily during 1960 and 1961. Resistance to tetracyline varied, but in some years 80% or more of strains tested were sensitive. Resistance to streptomycin increased, all strains being sensitive in 1959, but less than half from 1965 onwards. All but two of 867 strains tested were sensitive to chloramphenicol and all but 12 of the 929 strains tested with nitrofurazole were sensitive. All strains tested were sensitive to kanamycin until 1966 when four resistant strains of colicine type 0 were isolated in a family outbreak (Thomas & Datta, 1969). Subsequent strains were sensitive until the end of 1967, when a school outbreak began from which 43 kanamycin-resistant type-7 infections were identified (Thomas, Haider & Datta, 1972). Only 2 of 219 strains tested were resistant to nalidixic acid and these were in one family (Thomas & Datta, 1969). About a quarter of 130 strains tested were resistant to ampicillin.

Many cases were prescribed for by their family doctors, but medicine was not always taken as directed. However, 262 children who received supervised treatment with one of the drugs named in Table 4 were studied closely. *Sh. sonnei* persisted or recurred after treatment in 145 (55%) cases, regardless of the *in vitro* sensitivity to the drug used.

Table 6. *Sonne dysentery: annual number of GP index cases and MOH index infections in* 18 *primary schools in Old Edmonton*

School reference	No. of pupils	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	Total
S 2	298	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S 3	436	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	2
S 10	175	—	—	—	—	—	—	—	1	—	—	—	—	—	—	2	—	3
S 5	87	—	—	—	—	—	—	—	—	—	3	—	—	—	—	—	—	3
S 18	239	—	—	—	—	—	—	—	—	—	5	—	—	—	—	1	—	6
S 13	301	1	—	—	—	—	—	1	—	—	2	1	—	—	—	—	13	18
S 14	361	2	2	—	2	—	—	2	4	—	2	—	—	—	—	6	—	20
S 12	448	—	—	—	—	—	—	2	2	—	1	17	1	6	—	1	—	30
S 9	471	—	—	—	—	—	—	3	20	—	3	1	—	—	10	1	1	39
S 16	255	2	—	—	—	—	—	6	—	—	15	3	2	—	1	1	14	44
S 19	500	—	2	1	38	—	—	2	2	—	1	—	—	1	—	1	—	48
S 6	560	1	—	13	2	—	—	3	23	3	4	3	—	1	—	10	2	65
S 1	932	—	—	—	—	—	—	—	3	—	—	2	1	64	1	2	1	74
S 11	482	—	—	—	—	—	—	1	—	—	—	—	65	9	—	—	—	75
S 17	357	16	—	—	—	—	—	2	3	—	52	1	—	2	—	4	2	82
S 8	346	—	—	—	—	—	—	1	36	—	51	—	—	7	—	17	3	115
S 4	466	—	24	—	1	—	—	1	64	—	19	—	—	—	—	2	23	134
S 15	773	—	2	—	3	—	—	37	8	—	34	1	2	—	9	56	1	153
Total	7487	22	30	14	46	—	1	61	166	3	192	29	72	90	21	104	60	911

Bacteriological clearance and intermittent excretion

Intermittent excretion was recorded in 15 % of all index cases (264 of 1715). However, 122 of these cases had only a single intervening negative specimen liable to have been collected too soon after antibacterial therapy. Within the survey 745 child index cases were followed up to three or more concluding clearance specimens. Intermittency was recorded in 157 (21 %). In 92 (12 %) there was a single negative and in 60 of these it was the first specimen to follow diagnosis. There were two or more intervening negatives in the remaining 65 (9 %). Only 162 adults were followed to three clearance negative specimens. Intermittency was recorded in 14 (9 %) and in 9 of these there was a single negative from the specimen following diagnosis. There was no sex difference in the occurrence of intermittent excretion. For bacteriological clearance the aim was three consecutive negative specimens, which should have given complete clearance in over 90 % of cases.

Duration of infection in GP index cases

The duration of infection was taken to be from the onset of diarrhoea to the point midway between the last positive and the first negative specimen of a clearance series. Table 5 shows the duration in 1435 (84 %) of the 1715 index cases which were followed to two or more terminating negative specimens. Infection lasted less than 2 weeks in 47 % of 870 cases followed to three or more negatives and in 44 % of 565 cases followed to two negatives. In both groups 23 % of infections lasted more than 4 weeks, including 3 % which persisted longer than 8 weeks. It appears from this table that infection persisted longer in children than in adults. However, some long infections may have been missed in adults since follow-up was incomplete in 89 (25 %) of 365 adult index cases as compared with 191 (14 %) of 1359 child index cases. Adults were not compelled to send in follow-up specimens unless their occupation carried a public-health risk.

Seven infections lasted more than 4 months. These were 18 weeks in a woman aged 81 years, 18 and 21 weeks in girls aged 4 and 2 years respectively, 19 weeks in a girl aged 4 years who also had a Giardia infection, 17 weeks in a boy aged 7, 23 weeks in a boy of 15 sent home from boarding school, and 30 weeks in a boy aged 8 years.

Source of infection

More than half the index cases were pupils at primary schools. A special study was made of schools within the old Edmonton district of Enfield. This area had an average population of 7487 children of primary-school age, which was about 39 % of children of that age group in Enfield. During the 16 years of the survey, 17 of the 18 Edmonton primary schools studied here had bacteriologically confirmed cases of dysentery with a total of 911 children infected. Table 6 shows the annual incidence of GP index cases and MOH index infections in these schools.

An outbreak was defined as five or more proven infections in one term and a large outbreak as ten or more. It is clear that even in years of high incidence (e.g. 1960, 1962) large outbreaks affected only a few schools and most escaped with a few

Table 7. *Colicine typing of samples of* Sh. sonnei *strains tested,* 1961–1968

Colicine type	1961	1962	1963	1964	1965	1966	1967	1968	Total (%)
Type 0	6	8	52	33	69	18	63	42	291 (61%)
Type 7	—	2	26	13	26	—	1	43	111 (23%)
Type 2	2	—	2	7	—	1	6	—	18 (4%)
Type 6	—	1	15	—	2	—	—	—	18 (4%)
Type 4	—	6	5	—	3	—	—	—	14 (3%)
Type 13	—	1	8	2	1	—	—	—	12 (3%)
Type 11	—	—	2	1	1	—	7	—	11 (2%)
Type 1B	—	—	—	—	—	—	—	1	1
Type 3	—	—	—	—	1	—	—	—	1
Type 3A	—	—	1	—	—	—	—	—	1 (1%)
Type 8	—	—	1	—	—	—	—	—	1
Total tested	8	18	112	56	103	19	77	86	479 (100%)

sporadic cases or none at all. The largest proportion of pupils with proven index infection during an outbreak at any of these schools was 15%. The ten secondary schools with 4382 pupils had no dysentery outbreaks and individual cases in this age group were usually in contacts of infected primary-school children.

In one day-nursery which was open in Edmonton throughout the survey, a total of 95 cases were confirmed. The nursery accommodated 55 children. There were four considerable outbreaks during the survey, the largest of which affected 28 (51%) of infants attending.

Nineteen GP index cases of Sonne dysentery were thought to have been contracted abroad. Four of the five index cases of Flexner dysentery had recently been abroad. These were a Flexner 4A from South Africa, a Flexner 6 from Spain, a Flexner 2A from Yugoslavia and an untyped strain from Canada. These strains were studied by the late Dr K. P. Carpenter at Colindale.

Colicine typing

Four hundred and seventy-nine strains of *Sh. sonnei* were colieine-typed from 1961 onwards (Table 7) – 61% (291) were type 0 (non-colicinogenic) and 23% (111) were type 7. Five strains from infections contracted abroad were tested, of which two were type 2 and three were type 0.

Repeated infections

Second infections with *Sh. sonnei* were observed, sometimes after quite short periods of time. For example, during 1954–6 two nurseries suffered two distinct epidemics of dysentery each, separated by clear intervals of 5 and 18 months. Cases and contacts were followed up until three or more consecutive negative specimens had been obtained. Altogether 46 children and one adult were found to have been infected twice within 20 months. At one nursery 11 children were infected twice, 7 were ill in the first outbreak and two of these 7 were ill in the second outbreak 5 months later, while 4 were symptomless on each occasion. At the other nursery 6 children were infected twice, 3 were ill during the first outbreak and 4, including these 3, were ill during the second outbreak 18 months later, while 2 remained well

Table 8. *Infection rates among household contacts of Sonne dysentery*

Age of index case	Age and sex of exposed household contact	No. of household contacts	
		Tested	Found infected
Child	Child		
	Male	1728	765 (44%)
	Female	1692	711 (42%)
	Adult		
	Male	1799	286 (16%)
	Female	2118	525 (25%)
Adult	Child		
	Male	230	67 (29%)
	Female	205	85 (41%)
	Adult		
	Male	290	44 (15%)
	Female	264	48 (18%)
All	All exposed children	3855	1628 (42%)
	All exposed adults	4471	903 (20%)

NOTE. There were a further 20 infections found among 63 household contacts tested whose sex or age was not recorded.

during both infections. Among infected household contacts 18 of 28 children were reported to be ill on the first occasion and 9 on the second. The one adult was symptomless throughout.

The median duration of excretion was shorter in the second infection, being 11 days for the second compared with 24 days for the first infection. However, the interpretation of this comparison is in doubt because the children were older and tetracycline rather than sulphonamide was used to treat the later cases.

Multiple infection

Fifty-two of the 2535 Sonne dysentery index infections were found to harbour other pathogens. These included 29 *Giardia*, 8 *Salmonella* and 7 enteropathogenic *E. coli* infections.

Household infection

Specimens of faeces were collected from other members of the households of 2397 (95%) of GP index cases and MOH index infections. Of these 2397 households 1380 (58%) were found to contain further infected persons, a few with double infections, many without symptoms. The average number of additional persons tested in each household was 3·6 where the index case was a child and 2·8 where the index case was adult. The contact infection rate also tended to be higher when the index case was a child. Infection was higher among contact children (42%) than adults (20%) and among adult females (24%) than males (16%).

Table 9. *Sonne dysentery and salmonellosis compared: features of the two Enfield studies*, 1953–1968

A. TOTAL INFECTIONS

	No. found 1953–68		Annual incidence per 10,000 population	Percentage of new cases of diarrhoea
	Persons	Households		
Sonne dysentery	5319	2620	12·1	10%
Salmonellosis	917	580	2·1	2%

B. INDEX CASES (GP)

	Average no. per year	Range	Annual incidence per 10,000 popn.	Age and sex	Seasonal variation	Severity of illness	Duration of infection	Intermittent excretion
Sonne dysentery	107	16–293	3·9 (children 15·8 adults 1·0)	Many more children than adults (3·8:1) more female than male adults. Primary-school children affected twice as often as pre-school children	A winter excess	4% were admitted to hospital and just under a half of the remainder had blood or pus in their stools	23% of infections lasted more than 4 weeks and 3% more than 2 months. Children may have had slightly longer duration than adults	15% of cases
S. typhimurium	23	5–39	0·8 (children 2·5, adults 0·4)	More child than adult cases (1·4:1) and more males than females. Twice as many cases of pre-school as primary-school age	A summer excess until 1964	13% were admitted to hospital and half the remainder had blood or pus in their stools	50% of infections lasted more than 4 weeks and 21% more than 2 months. Children had slightly longer duration than adults	20% of cases

Table 9 (*cont.*)

B. INDEX CASES (GP) (*cont.*)

	Average no. per year	Range	Annual incidence per 10,000 popn.	Age and sex	Seasonal variation	Severity of illness	Duration of infection	Inter-mittent excretion
Other salmonellas	11	3–27	0·4 (children 0·8, adults 0·3)	Fewer child than adult cases (0·7:1) and slightly more females than males. Twice as many cases of pre-school as primary-school age	A summer excess	17% were admitted to hospital and just under a third of the remainder had blood or pus in their stools	57% of infections lasted more than 4 weeks and 26% more than 2 months. Children had slightly longer duration than adults	20% of cases

C. SPREAD OF INFECTION

	Source of infection	Infection of household contacts
Sonne dysentery	Over half the index cases were children attending primary schools, many arising during outbreaks. Outbreaks were reported in nurseries, but not secondary schools. 1% of index cases probably became infected during foreign travel. Case-to-case spread was common.	58% of households had infected contacts and 30% of household contacts became infected (42% of child and 20% of adult contacts).
Salmonellosis	Less than a fifth of index cases were of primary-school age. 8% of cases arose in known food-borne outbreaks and 3% were probably contracted abroad. The remainder were sporadic household episodes among those sharing foodstuff. Evidence of case-to-case spread was rare.	36% of households had infected contacts and 18% of household contacts became infected (24% of child and 16% of adult contacts).

Dysentery and salmonellosis compared

Table 9 summarizes findings in the concurrent surveys in Enfield during the years 1953–68. The salmonella study has been reported (Thomas & Mogford, 1970).

The incidence of *Sh. sonnei* was 6 times that of salmonella infection, with a much higher proportion of child cases and infections. More than 4 times as many households were infected by *Sh. sonnei*, and case-to-case spread was evident. Most Sonne infections arose from outbreaks in primary schools. Salmonellosis did not spread in schools, but was more often associated with infected foodstuffs, especially in the summer season. Little evidence of case-to-case spread was found. More of the salmonella than of the Sonne dysentery infections were associated with severe illness and prolonged excretion. Intermittent excretion was seen in 20% of salmonella and 15% of *Sh. sonnei* infections. Cellular stools were more common in *Salmonella typhimurium* infections than in other salmonella or Sonne infections. In several respects *S. typhimurium* occupied a position intermediate between 'other salmonellas' and *Sh. sonnei*.

DISCUSSION

This 16-year study of shigellosis in family practice in North London was made concurrently with our investigation of salmonellosis. It had the same advantages of a reasonably stable community, the co-operation of family practices in the same district over many years, the active participation of the public-health department throughout, and co-ordination by a single laboratory of the Public Health Laboratory Service.

During the period concerned, 1953–68, an average of about 30,000 cases of bacillary dysentery and 30 deaths were notified each year in England and Wales. There has been an unexplained sharp fall in notifications since 1969, although there has not been as great a fall in deaths. Meanwhile, however, the diagnoses of dysentery and gastroenteritis, taken together, recorded by the Royal College of General Practitioners since 1967 have shown an increase.

There was a remarkable general fall in dysentery notifications in England and Wales after the war, but this trend was reversed by 1950. In the 5 years preceding this study Sonne dysentery had at first been a rare disease in Enfield – only four cases being diagnosed from 1948 to November 1950. Then an increasing number of school and nursery outbreaks arose. During the years of survey, 1953–68, *Sh. sonnei* accounted for 10% of all new diarrhoea cases investigated, whereas salmonellosis accounted for 2% and only six *Sh. flexneri* infections were seen. No other shigella species was encountered. In England and Wales at this time *Sh. flexneri* represented 2·1% of the total dysentery isolations reported by the PHLS, but in some British cities studied from 1962 to 1966 by Hunponu-Wusu (1970) Flexner dysenteries were common – in Birmingham 13% and in Glasgow 28% of dysentery isolations.

The liability to *Sh. sonnei* infection and to gastroenteritis was greater in childhood than in adult life. The incidence, already high in the pre-school age group, rose greatly in primary-school children and then fell sharply in secondary-school

children and was lower still in adults, where it was only a little higher than that of salmonellosis. Repeated infection was noted in several families and in index cases at two day-nurseries. It appears that immunity to infection and to disease can be very short-lived. The duration of immunity after oral vaccination has not yet been fully assessed (Mel *et al.* 1971; Linde, Koch & Urbach, 1972; W.H.O., 1972).

We found little evidence of case-to-case spread of salmonellosis. In contrast there was evidence of a considerable amount of case-to-case spread of Sonne dysentery within primary schools and nurseries, but *not* in secondary schools, where sporadic cases were the rule. Multiple infections were found in 58% of households and in many of these a clear history of successive cases of clinical dysentery was available. Nearly twice as many adult females as males were found to be infected in households where the index case was a child. This we assume to indicate the greater exposure to infection of the mother of young children. These proportions were not unlike those reported by Hollins (1970), particularly with regard to the high incidence in primary-school children.

In contrast with our observation that salmonella infection showed a summer predominance until 1965, *Sh. sonnei* infection tended to follow the school year, with its large September intake of susceptible 5-year-olds. A few cases in October were often followed by outbreaks later in the winter. Since epidemics were not found to be associated with a uniform rise in incidence, but were the sum of localized outbreaks, it would appear that the early detection and limitation of such outbreaks could prevent large epidemics.

Sharp (1972) has observed that undernotification is common. Notifications for Enfield included a proportion of cases which ordinarily remain undiagnosed, especially during epidemics. Reid (1969) indicated the importance of engaging the support of the local population to identify cases and carriers. The Enfield policy involved the co-operation of the Medical Officers of Health with school teachers in the identification of cases and the supervision of strict hand and toilet hygiene (Thomas, 1966). It has been reported that about two-thirds of children become infected in uncontrolled primary-school outbreaks (Annotation, 1966). The highest incidence of index infections in any school in this study was 15% of the school population – a figure which does not include infected siblings.

It became clear that outbreaks in primary schools and nurseries were the foci of dysentery dissemination and that the control of these outbreaks contributed to the health of the community. In this survey only a small proportion of pre-school children attended nurseries, but even among this small number many cases of dysentery were observed. No outbreaks were related to a particular foodstuff. The school meals staff in the area were screened regularly for intestinal infections, but *Sh. sonnei* was very seldom found except during outbreaks in the school concerned. Perhaps infected catering staff, initially victims of an outbreak, may have helped to extend some of the outbreaks.

Some family doctors prescribed antibacterial drugs for cases of gastroenteritis. Knox (1972) concluded that antibiotic treatment made no difference to bacterial clearance of shigella infections. We commonly recovered *Sh. sonnei* from patients after treatment. Like Farrant & Tomlinson (1966) we observed changes in anti-

biotic sensitivity. By the end of the survey most strains of *Sh. sonnei* were resistant to sulphonamides and streptomycin, many to tetracycline and ampicillin; but very few to chloramphenicol, nitrofurazole and nalidixic acid. The first strains in England resistant to nalidixic acid and to kanamycin were reported (Thomas & Datta, 1969) and an outbreak of kanamycin-resistant infection was observed (Thomas *et al.* 1972).

Sh. sonnei infection was less prolonged than salmonella infection – a quarter of the cases as compared with a half lasted longer than 1 month and 1 % as compared with 10 % longer than 3 months. Excretion may have persisted longer in children than in adults. There was no card index of cases before 1953 available for analysis, but it may be interesting to record that during 1951 and 1952 a median duration of 26 days for *Sh. sonnei* infection in a group of 292 untreated children was compared with that of 23 days among 148 given sulphonamides. These median spans were longer than those of about 17 days observed in children throughout the period 1953–68, but the distribution of duration in the earlier cases is not recorded. If this difference is real it may possibly be explained by changes in immunological experience, infecting strains, or treatment. In 1944 Fairbrother found that half of all cases were still positive 2 weeks after onset and 30 % were still positive at 1 month.

The traditional view that a single negative is not sufficient for clearance was again substantiated. Hollins (1970) reported intermittent negatives in 17 % of cases, which compares with the 21 % we found among child index cases. Three consecutive negatives appear to have provided a practical margin of safety. Among children 9 % were observed to relapse after two negatives, and so it appeared that three negatives should have given complete clearance for over 90 % of infections. The amount and virulence of infection offered by intermittent excreters may well be less than from regular excreters (Andreeva *et al.* 1972), but there are circumstances in which they pose a danger. In residential institutions three negatives might not be sufficient and four or five would be safer.

A smaller proportion of *Sh. sonnei* than of salmonella index cases were admitted to hospital. Paradoxically, cellular dysenteric stools were seen rather *less* frequently in Sonne dysentery than in salmonella infections.

Sh. sonnei and salmonella infections were compared in the same population over the same period of time. The Sonne dysentery infections were somewhat briefer and milder, but their much higher incidence and infectivity and their liability to cause repeated infection pose a public-health problem which is at least as great as that of salmonellosis.

This study was made possible by the co-operation of General Practitioners, Medical Officers and many individual members of the Health and Education Departments, also the Head Teacher and staff of many schools in Enfield and in the old boroughs of Southgate and Edmonton. Our thanks are due to them and to the technical staff of the one-time Edmonton Public Health Laboratory, especially the late J. H. Cowlard, and to Dr T. M. Pollock for much help with the report.

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California encephalitis virus endemicity in the Yukon Territory, 1972

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SUMMARY

Sera from 218 of 1574 (14%) small mammals collected in the Yukon Territory between 14 May and 13 August 1972 neutralized a Yukon strain of California encephalitis virus (snowshoe-hare subtype). These included 133 of 319 (42%) snowshoe hares (*Lepus americanus*), 84 of 1243 (7%) ground squirrels (*Citellus undulatus*) and 1 of 12 (8%) tree squirrels (*Tamiasciurus hudsonicus*). California encephalitis virus (snow-shoe hare subtype) was isolated from four pools of unengorged *Aedes communis* mosquitoes collected near Whitehorse (61° N., 135° W.) and on one occasion each from pools of the same species collected at Hunker Creek (64° N., 138° W.) and at mile 125, Dempster Highway (66° N., 138° W.) during July 1972. Replication of a Yukon strain of California encephalitis virus was observed in wild-caught *Culiseta inornata* and *Aedes canadensis* mosquitoes after intrathoracic injection and holding at temperatures of 80°, 50° and 40° F.

INTRODUCTION

Confirmation of the endemic prevalence of California encephalitis (CE) virus in the Yukon Territory near Whitehorse (61° N., 135° W.) was first obtained during the summer of 1971 by the isolation of strains of CE virus (Montana snowshoe-hare subtype) from 12 of 84 pools of *Aedes canadensis* mosquitoes (McLean *et al.* 1972). This followed the initial demonstration of CE virus activity in mosquitoes and snowshoe hares (*Lepus americanus*) at comparable subarctic latitudes in east-central Alaska after ten human cases of infection of the central nervous system with CE virus were encountered during the spring of 1970 (Sudia, Newhouse, Calisher & Chamberlain, 1971; Feltz *et al.* 1972).

Within British Columbia, CE virus prevalence was suggested by detection of neutralizing antibodies in sera from human residents (Kettyls *et al.* 1972) at Dawson Creek (56° N., 120° W.), and in snowshoe hares or other mammals at Dawson Creek and Williams Lake (52° N., 122° W.) (McLean *et al.* 1971), at Kamloops (51° N., 120° W.) (Newhouse, Burgdorfer, McKiel & Gregson, 1963) and at Penticton (49° 30′ N., 120° W.) (McLean *et al.* 1970, 1971).

Isolation of CE virus from *A. canadensis* and *A. vexans* mosquitoes at Penticton (McLean *et al.* 1970) during the summer of 1969 provided the first definite evidence

of CE virus prevalence in British Columbia at a site some 300 miles north-west of the Bitter Root valley, Montana (46° N., 114° W.), where the snowshoe-hare subtype was isolated initially from the blood of *L. americanus* in 1959 (Burgdorfer, Newhouse & Thomas, 1961), and subsequently from *Aedes fitchii* and *Culiseta impatiens* mosquitoes (Newhouse, Burgdorfer & Corwin, 1971).

Within the boreal forest of northern Alberta near Rochester (54° N., 113° W.), strains of the Jamestown Canyon and snowshoe-hare subtypes of CE virus have been isolated from *Aedes communis* and *A. stimulans* mosquitoes during 1964 and 1965 (Iversen *et al.* 1969). On the prairie portions of southern Alberta near Brooks (50° N., 112° W.), two pools of *Culiseta inornata* mosquitoes yielded the snowshoe-hare subtype of CE virus in 1965 (Morgante & Shemanchuk, 1967).

The prototype strain of CE virus, termed BFS-283, was first isolated from *Aedes melanimon* mosquitoes in a hot irrigated area of the San Joaquin valley, California, during 1943 (Hammon & Sather 1966), more than 1000 miles south of British Columbia and some 2000 miles south of the Yukon. No more CE virus was found in the San Joaquin valley until 1963, when the Jerry Slough strain, BFS-4474 was isolated. Within North America between 1958 and 1964 an additional six serological subtypes of CE virus have been isolated from mosquitoes or man. Consistent but minor antigenic differences have been demonstrated between these strains, principally by immunodiffusion (Sudia *et al.* 1971), but also by complement fixation (Hammon & Sather, 1966). The prevalence of each subtype is confined principally to one geographical area within North America. During the past quarter century, 519 cases of aseptic meningitis or other clinically manifest infections with CE virus, affected human residents of the United States. The incidence increased from 3 cases in 1945 to 90 cases in 1970 (Sudia *et al.* 1971).

This paper reports additional isolations of CE virus strains from wild-caught mosquitoes both in southern and northern portions of the Yukon Territory during summer 1972, together with serological results on small forest rodents collected in the same areas, in an attempt to define more precisely the natural chains of infection of CE virus in a sub-Arctic region.

MATERIALS AND METHODS

Field mosquitoes

Mosquitoes were collected by hand-aspirators at five locations throughout the Yukon Territory between 11 June and 31 July 1972. Unengorged female mosquitoes were pooled in batches of 50–100, sealed in glass tubes in the field, placed immediately in dry ice at −70° C., and air-freighted to Vancouver once weekly, where they were held at −70° C. until tested for virus content. After identification by low-power microscopic examination of every mosquito, pools comprising 50–100 of the same species were ground in mortars suspended with 2·0 ml. diluent,* centrifuged at 2000 rev./min. for 3 min. to deposit coarse particles, and the super-

* The diluent for all virus titrations comprised Earle's saline with lactalbumin hydrolysate 0·5%, yeast extract 0·1%, neomycin 100 μg/ml., and Amphotericin B 5 μg/ml (medium ELY), to which was added 20% newborn-calf serum.

natant assayed for virus content by intracerebral injection of baby mice aged 1–3 days.

Live mosquitoes were placed in round cardboard cages in the field, chilled to about 50° F. and air-freighted to Vancouver, where they were held at 70 ± 2° F. until used for infection experiments.

Infection of mosquitoes in the laboratory

Mosquitoes were anaesthetized with carbon dioxide and injected intrathoracically with 0·003 ml. virus suspension in sterile water, using a finely drawn hard-glass pipette. Mosquitoes were placed in cylindrical cages, pledgets soaked in 15% sucrose solution were added, and the cages were inserted into sealed plastic bags to ensure high humidity. Mosquitoes were held at 80, 50, 40 and 30° F. until examined 2–60 days later for virus content by intracerebral inoculation of weaned mice aged 3–4 weeks.

Mammals

Small wild rodents and lagomorphs were collected by shooting principally within 50 miles of Whitehorse, Y.T. (61° N., 135° W.), but also surrounding Dawson City between Hunker Creek (64° N., 138° W.) and mile 125 on the Dempster Highway (66° N., 138° W.). Blood was collected from shot animals after extravasation into the pleural cavities. After separation of sera from clots at the field station, both samples were held at −20° C. and air-freighted frozen to Vancouver once weekly.

Virus isolation and typing

Brains of baby mice which developed encephalitis after intracerebral injection were removed aseptically, ground in mortars, extracted with 0·5 ml. diluent per brain, centrifuged at 2000 rev./min. for 3 min., and 0·03 ml. volumes of serial tenfold dilutions of supernatant were inoculated intracerebrally into mice aged 3 weeks. The 50% lethal dose for mice (LD 50) was calculated by the method of Reed & Muench (1938). Equal volumes of sodium deoxycholate 1/500 were added to virus suspensions containing at least 10^4 mouse LD 50 per 0·03 ml., held at 25° C. for 30–60 min., diluted 1/100 and injected intracerebrally into mice. Controls contained virus plus deionized water in place of sodium deoxycholate. Antisera prepared in rabbits or guinea-pigs to arboviruses including a Yukon 1971 CE isolate (McLean *et al.* 1972) were mixed in 0·1 ml amounts with equal volumes of virus diluted to contain 10^2 mouse LD 50 per 0·03 ml., held for 30–60 min. at 25° C., and injected into mice.

Serological tests

The neutralizing antibody content of all sera was determined by intracerebral injection of weaned mice with mixtures of undiluted sera plus a dilution of the Marsh Lake 23 strain of CE virus containing 50–100 mouse LD 50 per 0·03 ml. This strain constitutes the prototype of the 12 CE strains isolated from Yukon mosquitoes during 1971 (McLean *et al.* 1972). Sera which inhibited haemagglutination by Powassan (POW) or western equine encephalomyelitis (WEE) viruses were

examined in mouse neutralization tests against 50–100 mouse LD50 of the respective agent.

All sera were tested simultaneously for haemagglutination inhibition (HI) antibodies to 4–8 agglutinating doses of POW (L.B strain) and WEE (McMillan strain). Sera were extracted twice with acetone, absorbed with goose erythrocytes, and serial twofold dilutions were made in borate saline pH 9 containing 0·4 % bovalbumin. Disposable Microtiter plates were employed throughout. Haemagglutinins were prepared by extraction of infected baby-mouse brains with sucrose and acetone (Clarke & Casals, 1958). Dilutions containing 4–8 agglutinating doses per 0·025 ml. were added to each cup. After holding serum–virus mixtures for 1 hr. at 25° C., each cup received 0·05 ml. amounts of 0·25 % suspensions of goose erythrocytes in appropriate virus-adjusting diluents. The haemagglutination patterns were read after incubation for 1 hr. at 25° C. for POW and 37° C. for WEE, usually at pH 6·4 for both viruses.

Complement-fixing antibodies to CE virus (Marsh Lake 23 strain), using optimal dilutions of borate-saline extracts of infected baby-mouse brains as antigens were sought in all sera after preliminary screening for anticomplementary activity after dilution 1/2·5 in 0·15 M saline. Positively reacting sera were titrated for their antibody contents, using Microtiter plates.

RESULTS

Mosquito isolations

Strains of California encephalitis virus were isolated from 6 of 148 pools of unengorged female mosquitoes, totalling 10,317 insects, which were collected at five Yukon locations between 11 June and 31 July 1972 (Table 1). All six strains were derived from 9048 *Aedes communis* which were tested in 125 pools. This gave an overall minimum field infection ratio (MFIR) (Sudia *et al.* 1971) of 1:1508. Virus isolations were confined to Marsh Lake with MFIR 1:668, Hunker Creek with MFIR 1:1654 and Dempster Highway mile 125 with MFIR 1:2854. Virus was first detected in mosquitoes at Marsh Lake on 3 July and the final isolations were obtained there on 31 July. Isolations from the two northerly areas were obtained only during late July.

Each isolate induced encephalitis 3–4 days after intracerebral inoculation of baby mice, whose brains yielded about 10^6 LD 50 per 0·03 ml. upon titration intracerebrally in weaned mice. Infectivity was reduced at least 100-fold by treatment with sodium deoxycholate. All isolates were neutralized completely by antiserum to the Yukon 1971 prototype strain of CE virus (Marsh Lake 23), which was antigenically identical with the Montana snowshoe-hare subtype by immunodiffusion tests (C. E. Calisher, personal communication). Each Yukon 1972 strain was reisolated from mosquito suspensions after storage at − 70° C. for an additional 2–6 weeks after primary isolation.

Table 1. *California encephalitis virus isolations from Yukon mosquitoes*, 1972

Collection site	Species	Week commencing: June 11	June 18	June 25	July 2	July 9	July 16	July 23	July 30	Total: Ratio	Total: MFIR	Total: Mosq. tested
Tagish, 60½° N., 135° W.	*A. communis*	0/6	0/16	—	—	—	—	—	—	0/22	—	1622
	A. canadensis	—	0/2	—	—	—	—	—	—	0/2	—	98
Marsh Lake, 61° N., 135° W.	*A. communis*	0/6	0/7	—	1/9	1/13	0/2	—	2/3	4/40	1:668	2667
	A. canadensis	0/1	—	—	0/7	0/3	0/2	—	0/3	0/16	—	1012
	Cs. inornata	0/1	—	—	—	—	—	—	—	0/1	—	3
Lookout, 61° N., 135½° W.	*A. communis*	—	—	—	0/4	—	—	—	—	0/4	—	251
Hunker Creek, 64° N., 138° W.	*A. communis*	—	—	0/15	—	—	—	1/6	—	1/21	1:1654	1654
	A. canadensis	—	—	—	—	—	—	0/2	—	0/2	—	141
	Cs. inornata	—	—	0/1	—	—	—	—	—	0/1	—	9
Dempster Hwy., 66° N., 138° W.	*A. communis*	—	—	0/2	—	—	—	1/17	—	1/38	1:2854	2854
	Cs. inornata	—	—	0/1	—	—	—	—	—	0/1	—	6
Total	*A. communis*	0/12	0/23	0/36	1/13	1/13	0/2	2/23	2/3	6/125	1:1508	9048
	A. canadensis	0/1	0/2	—	0/7	0/3	0/2	0/2	0/3	0/20	—	1251
	Cs. inornata	0/1	—	0/2	—	—	—	—	—	0/3	—	18
	All	0/14	0/25	0/38	1/20	1/16	0/4	2/25	2/6	6/148	1:1719	10317

MFIR: minimum field infection ratio = (number of isolations)/(mosquitoes tested).

Table 2. *Replication of California encephalitis virus after injection of* Culiseta inornata

(Days of extrinsic incubation at stated temperatures.)

Mouse LD50 injected	Mosq. part	80° F.			50° F.			40° F.				
		5	12	19	7	30	48	5	12	19	43	60
300	SG	2·7*	2·3	3·0	0	1·5	1·9	0	0	2·8	2·0	2·7
	TH	4·0	3·8	4·7	2·2	2·9	1·9	1·5	2·3	3·0	2·3	2·7
30	SG	2·0	—	—	1·8	1·5	—	—	—	0	0	2·0
	TH	3·0	—	—	2·2	3·0	—	—	—	0	2·0	2·3
3	SG	2·0	2·3	—	0	2·3	—	—	0	0	1·8	—
	TH	3·7	1·8	—	1·8	3·0	—	0	0	0	1·5	—
0·3	SG	—	—	—	—	1·5	—	—	—	—	—	—
	TH	—	—	—	0	3·0	—	—	—	—	—	—
0·03	SG	—	—	—	—	1·5	—	—	—	0	—	—
	TH	0	0	—	0	2·0	—	0	0	0	—	—

SG, Salivary glands; TH, thorax.
* Mean log mouse LD50 of CE virus per mosquito part.

Table 3. *Replication of California encephalitis virus after injection of* Aedes canadensis

(Days of extrinsic incubation at stated temperatures.)

Mouse LD50 injected	Mosq. part	80° F.		50° F.					40° F.	
		2	6	5	6	11	17	23	5	17
3·0	SG	3·0*	2·0	0	0	—	2·0	—	0	1·8
	TH	2·3	3·5	0	2·0	—	3·5	—	0	2·0
3	SG	2·7	2·7	0	0	0	—	2·5	0	0
	TH	3·0	3·7	0	1·7	0	—	3·0	0	1·8
0·3	SG	—	1·5	0	0	0	—	—	0	0
	TH	—	3·0	0	0	0	—	—	0	0

SG, Salivary glands; TH, thorax.
* Mean $\log_{10}$ mouse LD 50 of CE virus per mosquito part.

Mosquito infection experiments

During preliminary mosquito collections at Marsh Lake on 15 May 1972, *Culiseta inornata* bit the senior investigator avidly, despite an atmospheric temperature below 32° F. This species therefore provided a model for examination of virus replication in mosquitoes at temperatures below 80° F., which is standard for transmission experiments involving arboviruses from tropical and subtropical areas. Wild-caught *Culiseta inornata* from the Yukon were maintained at 40° F. for 60 days and at 30° F. for 12 days. *Aedes canadensis* from the Yukon were maintained at 40° F. for 17 days. On the contrary, *Aedes aegypti* obtained from a Vancouver mosquito colony could not be maintained at temperatures below 50° F.

After intrathoracic injection of *Culiseta inornata* with 300, 30 and 3 mouse LD 50 of Marsh Lake 23 virus, substantial increments of infectivity were demonstrated both in salivary glands and thoraces of mosquitoes tested individually or in

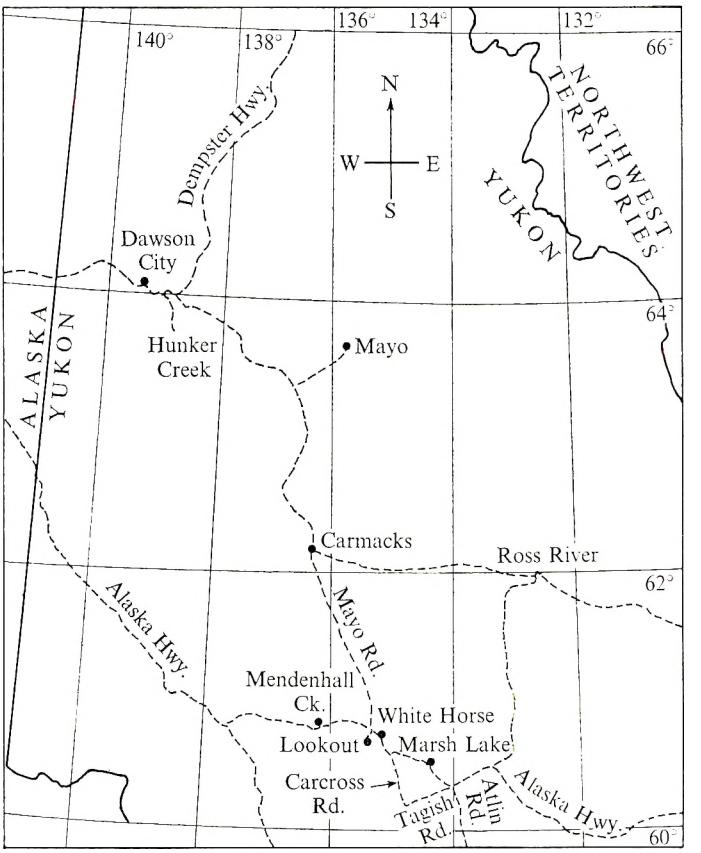

Fig. 1. Field collection sites in the Yukon Territory, summer 1972.

pairs after 5 days of extrinsic incubation at 80° F. Virus titres remained elevated as long as 19 days after injection of 300 mouse LD50 (Table 2). No infectivity was detected 12 days after injection of 0·03 mouse LD50. Following incubation at 50° F. substantial increments of virus content were observed in thoraces 30 days after inoculation of as little as 0·3 mouse LD50, and in each instance the titres increased above those observed at 7 days. However, salivary glands at 30 days contained relatively small quantities of virus. Mosquitoes which received 0·03 mouse LD50 contained only trace quantities after 30 days of extrinsic incubation. Virus replication was demonstrated after holding for 43 days at 40° F., following injection of 300, 30 and 3 mouse LD50. However, the time at which substantial increments of infectivity were first detected was prolonged considerably beyond that observed at 80° and 50° F.

After intrathoracic injection of *Aedes canadensis* with 30, 3 and 0·3 mouse LD50 of Marsh Lake 23 virus, substantial virus titres were detected in salivary glands and thoraces after 6 days of extrinsic incubation at 80° F. (Table 3). Minimal amounts of virus were detected solely in thoraces after 6 days incubation at 50° F. in mosquitoes which received 30 or 3 mouse LD50. Significant increments of titres both in thoraces and salivary glands were observed at 17 and 23 days respectively.

Table 4. *California encephalitis neutralizing antibody in Yukon mammals*, 1972

Location	May			June			July		August		Total species			Total All
	La	Cu	Th	La	Cu	Th	La	Cu	La	Cu	La	Cu	Th	
Lookout	4/12	7/47	0/1	13/36	6/65	—	15/36	—	1/4	—	33/88	13/112	0/1	46/201
Mayo Road	6/23	0/82	—	2/8	1/16	—	—	2/47	0/1	5/39	8/32	8/184	—	16/216
Carcross Rd	7/13	15/58	—	—	1/48	0/4	—	5/73	—	4/33	7/13	25/212	0/4	32/229
Marsh Lake	30/55	2/45	—	7/29	7/35	0/1	10/18	3/75	—	0/39	47/102	12/194	0/1	59/297
Mendenhall Creek	—	5/32	0/1	0/1	0/24	—	—	0/58	0/1	1/24	0/2	6/138	0/1	6/141
Tagish Road	—	3/61	1/2	1/11	3/82	0/3	—	1/73	2/4	0/23	3/15	7/239	1/5	11/259
Atlin Road	3/12	0/29	—	4/11	2/62	—	—	—	—	7/59	7/23	9/150	—	16/173
Hunker Creek	—	—	—	10/24	—	—	16/18	—	—	—	26/42	—	—	26/42
Dempster Hwy	—	—	—	—	—	—	2/2	4/14	—	—	2/2	4/14	—	6/16
Total no.	50/115	32/354	1/4	37/120	20/332	0/8	43/74	15/340	3/10	17/217	133/319	84/1243	1/12	218/1574
%	43	9	25	31	6	0	58	4	30	8	42	7	8	14

La, *Lepus americanus*; Cu, *Citellus undulatus*; Th, *Tamiasciurus hudsonicus*.

In mosquitoes incubated at 40° F., minimal quantities of virus were detected 17 days after injection of 30 and 3 mouse LD 50.

Mammalian serology

Between 14 May and 13 August 1972, sera were collected from 1574 wild mammals at seven general locations near Whitehorse and two areas near Dawson City (Fig. 1). Neutralizing antibodies to the Marsh Lake 23 strain of CE virus were found in 218 (14 %) animals (Table 4), including 133 of 319 (42 %) snowshoe hares (*Lepus americanus*), 84 of 1243 (7 %) ground squirrels (*Citellus undulatus*) and 1 of 12 (8 %) red squirrels (*Tamiasciurus hudsonicus*). The incidence of antibody in snowshoe hares was highest during July (58 %), but a substantial proportion (43 %) of hares collected during May neutralized CE virus. Antibody prevalence exceeded the overall mean of 42 % at Carcross Road (52 %), Marsh Lake (47 %), Hunker Creek (62 %) and along the Dempster Highway to mile 125 (100 %).

Complement-fixing antibodies to CE virus were found in 78 of 133 snowshoe hare sera which neutralized this agent, and in an additional 33 sera which were devoid of neutralizing antibody. Complement-fixing antibodies were also found in 7 of 84 sera from ground squirrels which neutralized CE virus, and in a further 15 without netralizing antibody.

Haemagglutination-inhibition antibodies to POW virus were detected in sera from 44 mammals, including 4 snowshoe hares and 40 ground squirrels, whilst WEE antibodies were found in 34 mammals, including 3 hares and 31 squirrels. Sera from 4 animals inhibited haemagglutination by both viruses. However, none of these sera neutralized their respective viruses.

Blood clots between 11 June and 29 July from 38 mammals devoid of CE neutralizing antibodies were examined for virus content by intracerebral injection of suckling mice. No virus was isolated.

DISCUSSION

Isolation of California encephalitis virus from *Aedes communis* mosquitoes collected along the Dempster Highway in the Yukon Territory at 66° N., 138° W., together with the demonstration of CE neutralizing antibodies in showshoe hares and ground squirrels from the same location, demonstrates clearly the existence of a natural endemic focus of CE virus near the Arctic Circle. These results extend northwards by two degrees of latitude the previously established northern limit of CE virus activity in North America near Tok, Alaska (64° N., 144° W.), about 150 miles west of Dawson City, Y.T. (Feltz *et al.* 1972; Sudia *et al.* 1971). Additional isolations from mosquitoes during 1972 confirm our 1971 demonstration of the endemic prevalence of CE virus at Marsh Lake (61° N., 135° W.) and other locations near Whitehorse (McLean *et al.* 1972). They substantiate our proposition that CE-neutralizing antibody in 22 % of mammals collected near Dawson City (64° N., 138° W.) arose from natural infection by this agent.

Although all six CE virus strains from mosquitoes during 1972 were isolated from

A. communis, in contrast to *A. canadensis*, from which all 12 strains were recovered during 1971, both these mosquito species have yielded strains of the snowshoe-hare subtype of CE virus in more southerly portions of western Canada (Iversen *et al.* 1969, McLean *et al.* 1970). The minimum field infection ratio of 1:2854 for *A. communis*, at the northern fringe of the boreal forest (66° N.) during 1972, approached that of 1:2168 for this species near Rochester, Alberta (54° N.), at the southern extremity of the boreal forest, where wooded areas merge into the Canadian prairie. Whilst the maximum prevalence of CE virus in the Yukon during 1972, in common with 1971, was found at Marsh Lake, where the MFIR was 1:668, the overall rate of 1:1508 for *A. communis* collected at all Yukon test sites compares favourably with the MFIR of 1:1518 for the principal vector species *A. fitchii* in the Bitter Root valley of Montana (Newhouse *et al.* 1971) where the snowshoe-hare subtype of CE virus was first isolated.

Populations of *A. communis* and *A. canadensis* became extremely abundant from mid-June to late July, when daytime temperatures usually exceeded 80° F. *Culiseta inornata*, however, was prevalent during May, when daytime temperatures were cooler, and it was observed to feed on humans even at temperatures below 32° F. The propagation of CE virus in *A. canadensis* and *Cs. inornata* following intrathoracic injection of 3–30 mouse LD50, at temperatures as low as 40° F., following extrinsic incubation periods 2–5 weeks longer than those encountered after incubation at 80° F., suggests that these mosquito species may provide both an effective overwintering mechanism in addition to serving as summertime natural vectors.

The high incidence of CE-neutralizing antibodies in sera from snowshoe hares collected in the Yukon during 1971 (25 %) and also 1972 (42 %) strongly suggests that *Lepus americanus* provides the principal vertebrate reservoir of infection, although a small proportion of ground squirrels, comprising 8 % in 1971 and 7 % in 1972, acquired antibody presumably following infection in nature. Isolation of the snowshoe-hare subtype of CE virus from the blood of *L. americanus* collected at Tok, Alaska (Feltz *et al.* 1972), Rochester, Alberta (Hoff, Yuill, Iversen & Hanson, 1969) and Hamilton, Montana (Burgdorfer *et al.* 1961), confirms the role of this species as a natural reservoir. The ability of chipmunks and squirrels collected in Wisconsin to develop viraemia, after peripheral inoculation with the serologically related La Crosse subtype of CE virus, at a titre sufficient to infect *Aedes triseriatus* mosquitoes which subsequently transmitted this agent by biting mice (Pantuwatana, Thompson, Watts & Hanson, 1972), suggests that the zoologically related Arctic ground squirrel, *Citellus undulatus*, may also serve as a vertebrate reservoir of CE virus in the Yukon.

Although serological surveys of small wild mammals revealed extensive evidence of infection both at southern and northern Yukon locations, the incidence of infection among the sparse human population of these areas remains undetermined through lack of serological investigation. However, in adjacent portions of Alaska near Tok, detection of CE neutralizing antibody in 72 % of 325 human residents during 1968 and the acquisition of HI antibodies by all seronegative subjects during the subsequent year, despite the onset of headache and fever in only 10 of 95

persons shortly before sero-conversion (Feltz *et al.* 1972), strongly suggests the occurrence of mild or subclinical infections with CE virus tangentially to the natural cycle of infection between mosquitoes and rodents in sub-Arctic regions of Alaska and the Yukon Territory.

California encephalitis virus appears to be the sole arbovirus which is at present prevalent in the Yukon Territory, where *Aedes* and *Culiseta* mosquitoes assume high summertime population densities, but *Culex tarsalis* has not been collected. Although WEE neutralizing antibodies suggesting past virus infections have been detected in 8 of 160 reindeer collected as far north as Atkinson Point, Northwest Territories (70° N., 132° W.), during 1968 (Burton & McLintock, 1970), no evidence of WEE infection has been detected in Yukon mammals during 1971 and 1972. The northern limit of *Culex tarsalis*, the principal natural vector of WEE on the Canadian prairies and in other portions of the western United States, is about 53° N. (Burton & McLintock, 1970). Usually *Aedes* mosquitoes alone are insufficient to maintain WEE virus in nature. In a transitional zone between prairie and boreal forest at Rochester, Alberta (54° N.), some serological evidence of WEE infection was detected in snowshoe hares during 1963 and 1965 when human cases occurred frequently throughout Alberta. However, only *Aedes* mosquitoes were collected at Rochester (Yuill, Iversen & Hanson, 1969). Possibly, snowshoe hares became infected tangentially to a natural cycle involving *C. tarsalis* mosquitoes in adjacent prairie portions of the terrain. The absence of WEE infection from Yukon mammals correlates well with the lack of collection of *C. tarsalis* in that region.

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Typing of *Pseudomonas aeruginosa*: comparison of the phage procedure with the pyocine technique

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SUMMARY

Two hundred and sixty strains of *Pseudomonas aeruginosa* were isolated from patients of the Asaf Harofe Government Hospital. The strains were typed by phage technique and 64 of them were also typed according to pyocine sensitivity. The two methods proved complementary, and reduced the number of untypable strains. Phage typing was performed with both routine test dilution (RTD) and more concentrated phage suspensions. The most reliable results were obtained at 100 RTD.

INTRODUCTION

Pseudomonas aeruginosa has become an agent of great importance in hospital-acquired infections during this decade. There may be several reasons for this, e.g. suppression of the normal intestinal flora by broad-spectrum antibiotics, therapeutic use of immunosuppressive drugs, the use of sophisticated medical instrumentation, and the presence of a larger number of at-risk individuals, especially infants and older people (Williams, Williams & Hyams, 1960; Ayliffe *et al.* 1965, 1966; Fierer, Taylor & Gezon, 1967; Hardy, Ederrer & Matsen, 1970). Many strains of *P. aeruginosa* are rather resistant to a wide range of antibiotic drugs (Lindberg, Curreri & Pruitt, 1970; Stone, Kalb & Joseph, 1971). In addition, *P. aeruginosa* seems to have an exceptional ability to survive and multiply in the hospital environment. *P. aeruginosa* has been recovered in hospitals from soaps, sinks, 'sterile' solutions, drinking water, and equipment for inhalation, as well as from food (Fierer *et al.* 1967; Moffet & Williams, 1967; Shooter, Gaga, Cook & Kumar, 1969; Weber, Werner & Matchnigg, 1971).

In order to locate successfully the source and vehicles of this infectious organism it is necessary to establish the relationship between all strains isolated. Three methods have been used to subdivide *P. aeruginosa* for epidemiological purposes: serotyping (Habs, 1957; Borst & De Jong, 1970), phage typing (Graber, Latta,

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Vogel & Brame, 1962; Sjöberg & Lindberg, 1968; Bergan, 1972), and pyocine typing (Wahba, 1963; Gillies & Govan, 1966; Govan & Gillies, 1969; Tagg & Mushin, 1971; Deighton, Tagg & Mushin, 1971).

The present communication is the first report from Israel on phage typing of *P. aeruginosa* strains. All isolates were from hospitalized patients. The results were compared with those obtained by pyocine typing.

MATERIALS AND METHODS

Organisms

Two hundred and sixty cultures of *P. aeruginosa* were isolated from 206 patients of the Asaf Harofe Government Hospital during the 9-month period from August 1971 to April 1972. The strains were identified by the following features: rapid positive oxidase reaction, production of pyocyanine and fluorescein, growth at 42° C. and absence of growth at 5° C.

Phage typing procedure

Phage typing was carried out with a set of 22 phages kindly supplied by Dr L. Sjöberg of the National Bacteriological Laboratory, Stockholm, Sweden. Growth of the propagating strains (PS) as well as propagation of phages was conducted on trypticase soy agar (TSA) as recommended by Sjöberg & Lindberg (1968). The routine test dilution (RTD) was determined as the highest dilution giving sub-confluent lysis. All strains were examined at RTD and at 100 RTD, and half of them were also examined at 10 RTD and at 1000 RTD. Final phage patterns were obtained at 100 RTD. Phage reactions were estimated as follows: 5+ : confluent lysis; 4+ : almost confluent lysis; 3+ : more than 30 isolated plaques; 2+ : more than 20 isolated plaques; 1+ : less than 20 isolated plaques. Only reactions of 3+ and above were regarded as positive for typing purposes. Inhibition reactions were characterized by thinning of the bacterial growth in the area where the phage had been applied. Each strain was typed 2–3 times in order to confirm reproducibility.

Pyocine typing

The procedure described by Tagg & Mushin (1971) and Deighton *et al.* (1971) was used.

RESULTS

Phage typing of 260 strains of *P. aeruginosa* at RTD gave 233 (88·9 %) typable and 29 (11·1 %) untypable strains. When typed at 100 RTD the number of typable strains increased to 240 (92·3 %). Half of the strains were also typed at 10 RTD and 1000 RTD. Typing at 10 RTD yielded less typable strains than did 100 RTD. Table 1 shows that typing at 100 RTD gave a better differentiation of the strains than did RTD. The phage pattern 73 (RTD) was composed of some strains untypable with pyocine and others of pyocine type 13—. At 100 RTD this difference corresponded to strains reacting with phage 352 and C 188 respectively. On the

Table 1. *Phage typing of* Pseudomonas aeruginosa *at different RTD values compared with pyocine typing*

Pyocine group	Phage patterns		
	RTD	10 RTD	100 RTD
UT	73	73	7/73/352
UT	73	73	7/73/352
UT	73	73	7/73/352
UT	73	7/73	7/73/352
13—	73	7/73/C188	7/73/C188
13−−	73	7/73/C188	7/73/C188
13—	73	7/73/C188	7/73/C188
13−−	73	7/73/C188	7/73/C188
31−−	44/1214/C11/M4	24/44/1214/C11/F8/M4	24/44/73/1214/C11/F8/M4
31−−	12/4/C11/M4	24/44/1214/C11/F8/M4	24/44/73/1214/C11/F8/M4
31−−	44/1214/C11/M4	24/44/1214/C11/F8/M4	24/44/73/1214/C11/F8/M4
31−−	7/73/352/M4	7/73/352/C18/M4	7/16/24/44/68/73/352/C18/F7/F8/M4
31−−	7/73/352	7/73/352/C18/F8/M4	7/16124/44/68/73/352/C18/F7/F8/M4
31−−	7/73/352/M4	7/73/352/C18/F8/M4	7/16/21/24/44/68/73/352/C18/F7/F8/M4
11−−	7/73/352/F8	7/73/352/C18/F8	7/24/44/73/109/1194/352/C11/C18/F8
34−−	21/68/73	7/21/68/73/352/C18/F8	7/21/68/73/352/C18/F8
3+−	7/68/73	7/68/73/352/C18	7/44/68/73/352/C188/F8
3++	44/1214	44/1214	44/1214/C11
10++	24/1214/M4	24/1214/C11/M4	24/1214/C11/M4
10++	44/1214/C11	44/1214/C11	44/1214/C11
10++	44/1214	44/1214	44/1214/C11
10++	44/1214	44/1214	44/1214/C11
1, UC(−+−+−)	44/1214	44/1214/C11	44/1214/C11
1, UC(−+−+−)	44/1214/C11	44/1214/C11	44/1214/C11
1, h	44/1214/C11	44/1214/C11	44/1214/C11
1, h	UT	44/1214/C11	44/1214/C11
1, h	UT	UT	44/1214/C11
1, h	UT	UT	44/1214/C11
5−−	UT	UT	21/44/1214
5−−	1214	44/1214	21/44/1214
5−−	1214	44/1214	21/44/1214
5−−	24/68/M4	21/24/68/73/M4	21/24/68/73/109/F8/M4
5−−	21/24/68/M4	21/24/68/73/M4	21/24/68/73/109/F8/M4
1, b	M6	M6	21/M6
1, b	M6	21/M6	21/M6
1, b	M6	21/M6	21/M6
1, b	M6	M6	21/M6

UT = untypable.

other hand, one of three strains reacting with pyocine 5−− was untypable with phages at RTD, whereas two others reacted with phage 1214. This difference disappeared when 100 RTD of the phage was used. Also some of the apparent lytic differences inside the pyocine 31 type disappeared at 100 RTD, and might have been artificial. By typing at 1000 RTD the number of typable strains was not increased above that obtained at 100 RTD, but in some cases almost all phages

Table 2. *Comparison of phage typing at* 100 *RTD with pyocine typing correlated with clinical data* (Pseudomonas aeruginosa)

Origin of isolate*	Ward	Date of isolation	Phage patterns	Pyocine group
U 1	44	14/9	21/F7	5^{--}
V 1	44	15/9	21/F7	5^{--}
P 2	93	3/9	21/24/68/73/109/F8/M4	5^{--}
P 2	93	8/9	21/24/68/73/109/F8/M4	5^{--}
P 2	93	9/9	21/24/68/73/109/F8/M4	5^{--}
R	42	26/8	21/44/1214	5^{--}
U 3	Amb.	4/11	21/44/1214	5^{--}
U 3	Amb.	9/11	21/44/1214	5^{--}
R	93	13/9	UT	1, h
P	93	19/9	44/1214/C11	1, h
R	37	22/9	44/1214/C11	1, h
R	15	12/10	44/1214/C11	1, h
R	28	8/11	44/1214/C11	1, h
R	40	31/8	44/1214/C11	1, h
R	37	1/11	44/1214/C11	1, h
R	37	10/11	44/1214/C11	1, h
R	15	12/10	44/1214/C11	1, UC($^{-+-+-}$)
R	37	13/10	44/1214/C11	1, UC($^{-+-+-}$)
P	42	7/10	44/1214/C11	17^{---}
R	27	17/8	44/1214/C11	17^{---}
R	28	23/8	44/1214/C11	10^{++}
R	40	16/9	44/1214/C11	10^{++}
B	44	30/9	44/1214/C11	10^{++}
R 4	15	5/10	21/68	10^{+-}
R 4	15	6/10	21/68	10^{+-}
R 4	15	7/10	21/68	10^{+-}
R 4	15	8/10	21/68	10^{+-}
U	93	30/8	UT	1, C
U	36	26/9	21/M6	1, b
U	36	28/9	21/M6	1, b
P	25	22/9	21/M6	1, b
B	37	22/9	21/M6	1, b
U	39	15/8	C18	1, b
U 5	36	23/12	C18/C188	1, b
P 5	93	9/12	C18/C188	1, b
P 5	93	10/12	C18/C188	1, b
P	30	9/12	C18/C188	14
B 6	26	26/9	7/73/C188	13^{--}
B 6	26	30/9	7/73/C188	13^{--}
B 6	26	1/10	7/73/C188	13^{--}
B	38	22/9	7/73/C188	12^{--}
R 7	42	13/9	7/73/352	UT
R 7	42	14/9	7/73/352	UT
R 7	42	15/9	7/73/352	UT
R	29	7/11	7/73/352	UT
R	28	31/12	UT	UT
P	42	7/9	2/7/21/68/73/352/C18/F8	UT
P	Opth.	7/9	2/7/21/68/73/352/C18/F8	34^{--}
U	43	4/8	2/7/21/68/73/352/C18/F8	3^{++}
U	30	25/10	2/7/21/68/73/352/C18/F8	3^{++}
R 8	30	5/9	7/16/21/44/68/73/352/C18/F7/F8/M4	29^{--}

Table 2 (*cont.*)

Origin of isolate*	Ward	Date of isolation	Phage patterns	Pyocine group
R 8	30	6/9	7/16/21/44/68/73/352/C18/F7/F8/M4	29^{--}
U 9	30	3/11	7/16/24/44/68/73/352/C18/F7/F8/M4	31^{--}
U 9	30	16/11	7/16/24/44/68/73/352/C18/F7/F8/M4	31^{--}
U 9	30	31/2	7/16/21/24/44/68/73/352/C18/F7/F8 M4	31^{--}
U 10	40	5/9	24/44/73/1214/C11/F8/M4	31^{--}
U 10	40	6/9	24/44/73/1214/C11/F8/M4	31^{--}
U 10	40	7/9	24/44/73/1214/C11/F8/M4	31^{--}
P	36	10/9	UT	2^{--}
P	93	5/11	UT	2^{--}
R 11	40	7/9	UT	UC8^{--}
R 11	40	10/9	UT	UC8^{--}
U	93	30/8	UT	UC^{--}(+++++−+−)
P	42	14/11	UT	UC^{+-}(++−++−++)

Each number indicates a specific patient with more than one isolation.
* Key to column 1: B = blood, P = pus, R = respiratory, U = urine, V = vaginal.

gave strong lysis reactions at 1000 RTD. The number of inhibition reactions also increased considerably. Final phage patterns will therefore be presented as obtained at 100 RTD.

Pyocine typing was performed on 64 of the strains. The strains could be divided into 18 distinct types including one group of untypable strains. The same strains could be divided into 12 fairly distinct phage patterns and nine untypable strains. Differences in two or more lytic reactions were considered significant. By a combination of both procedures the 64 strains could be divided into not less than 26 types, as shown in Table 2. Of nine strains which were untypable with phage, eight were typed by the pyocine procedure. On the other hand, five of the six strains which were untypable by the pyocine method gave phage patterns. Only one strain was thus found untypable by both pyocine and phage procedures (Table 2). It should be pointed out that this strain was biochemically a typical member of *P. aeruginosa*.

We examined a total of 260 strains of *P. aeruginosa* by phage lysis (Table 3). If again differences in two or more lytic reactions are accepted as significant, this strain collection can be divided into 22 patterns, including 22 strains resistant to lysis by phage. Seven strains gave different patterns on repeated typing, and were therefore designated in the table as miscellaneous.

Twenty-eight (48·2%) of the strains with phage pattern 44/1214/C11 were characterized by the development of a brown colour which usually started from the lysed area. This brown colour started developing in the incubator and its intensity increased when the culture was kept at room temperature for 5–6 hr. This reaction was typical and persistent when the strains were grown on TSA, whereas on synthetic medium (Sutter, Hurst & Fennia, 1963) the brown colour did not develop. In this survey the brown pigment was not observed among other strains.

Table 3. *Phage pattern distribution of* P. aeruginosa *isolated from* 206 *patients of Asaf Harofe Government Hospital from August* 1971 *to April* 1972

Serial No.	Phage patterns	No. of patients	Source of strains: Urine	Respiratory	Skin	Blood	Vaginal	Faeces	No. of strains	%
1	119	5	3	1	3	—	—	—	7	2·6
2*a**	1214	5	3	—	1	3	—	—	7	2·6
2*b**	21	12	—	7	3	—	5	—	15	5·7
3	21/F7	6	5	—	1	—	1	—	7	2·6
4	21/M6	12	4	5	2	2	1	—	14	5·3
5*	21/1214	2	2	—	—	—	—	—	2	0·7
6	21/31/C18	6	—	2	4	2	—	—	8	3·1
7	21/1214/109/1198	4	2	2	—	—	—	—	4	1·5
8	21/44/1194/C18/F8	3	2	—	1	—	—	—	3	1·2
9	21/44/1214	3	2	—	1	—	—	—	3	1·2
10	21/44/68/109/1194/C18/F8	1	—	—	—	1	—	2	3	1·2
11	21/24/68/73/109/F8/M4	3	1	1	3	—	—	—	5	1·9
12	24/44/73/1214/C11/F8/M4	2	3	—	—	1	—	—	4	1·5
13	C18/C188	19	7	8	6	—	1	—	22	8·2
14	7/73/352	7	3	6	—	—	—	—	9	3·4
15	7/73/C188	2	—	—	—	4	—	—	4	1·5
16	7/16/21/C18	8	5	1	2	—	—	—	8	3·1
17	7/21/24/44/109/1194	4	2	—	2	—	—	—	4	1·5
18	7/73/352/1214/C18/C21	3	6	—	—	—	—	—	6	2·2
19	7/16/21/24/44/68/73/352/C18/F7/F8/M4	18	17	8	2	1	1	—	29	11·0
20	2/7/44/68/73/352/C18/F8	4	2	—	2	—	—	—	4	1·5
21	44/C18/F7	5	—	—	5	—	—	—	5	1·9
22	44/1214/C11	45	13	27	17	—	1	—	58	22·4
23	Untypable	20	7	8	4	3	—	—	22	8·6
24	Miscellaneous	7	5	1	1	—	—	—	7	2·6
	Total	206	94	77	60	17	10	2	260	100·0
	%		36·1 %	29·6 %	23·0 %	6·5 %	3·8 %	0·8 %		

* 2a, 2b and 5 may belong to the same group.

DISCUSSION

According to the observations reported above, it is profitable to perform phage typing at 100 RTD of phage. The number of typable strains was higher than at RTD, there was a better differentiation of the patterns, and the homogeneity in reactions within certain types improved (Table 1). Sjöberg & Lindberg (1968) also found that the use of elevated concentrations of phage suspensions increased the number of typable strains, but with 1000 RTD they found a high incidence of inhibition reactions.

Typing of *P. aeruginosa* strains by two methods, such as pyocine typing and serotyping, has been proposed by Matsumoto, Tazaki & Kato (1968). However, several authors found serotyping of limited value as most strains fell into relatively few types (Hobbs, Gowland & Byers, 1964; Farmer & Herman, 1969). A combination of pyocine typing and phage typing was used by Farmer & Herman (1969). They first treated the strains with mitomycin C in order to obtain more distinct typing. By this procedure differences in sensitivity to the bacteriophages were frequently revealed; thus two strains which were epidemiologically identical varied in phage reactions, and phage lysis could therefore not be considered for typing. The typing method used in this study helped to overcome the limitations of each method. The fact that almost all isolates which were untypable by one method could be typed by the other might also be of some practical interest.

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Preservation of *Staphylococcus aureus* with unstable antibiotic resistance by drying

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SUMMARY

Cultures of *Staphylococcus aureus* showing unstable resistance to kanamycin, streptomycin, lincomycin and penicillin have been preserved by drying. Particular attention has been paid to organisms showing linked resistance to pairs of these antibiotics. Recoveries of viable cells from the desiccates was high and the proportion of resistant and sensitive cells was maintained both during storage and during heating. The preservation of the organisms on ceramic beads stored in air over silica gel promises to be an effective and convenient method for maintaining staphylococcal desiccates during studies of unstable antibiotic resistance.

INTRODUCTION

It is now well established that resistance to some antibiotics in *Staphylococcus aureus* is often controlled by extrachromosomal determinants (plasmids). Such resistance is often lost spontaneously and rapidly during growth and when maintained on ordinary culture media. Also in some studies, linkage has been demonstrated between different resistance factors and also between these factors and other metabolic markers. In investigations in this field it is an advantage to have readily available populations reliably characterized with regard to their content of resistant and sensitive cells. Although desiccation is a common means of preserving bacteria and could be expected to fulfil this requirement, there appears to be no published evidence to support this contention. This paper supplies some data on the subject and some also upon the effect of heat on such desiccates. The strains investigated were those previously shown to have linked and unstable antibiotic resistance to various combinations of kanamycin, lincomycin, streptomycin and penicillin (Grubb & Annear, 1972).

METHODS

Suspensions for drying

The organisms (Tables 1–3) were grown on nutrient agar and harvested after 20 hr. at 37° C. Heavy suspensions (10^{10} orgs/ml.) were prepared in sodium glutamate the concentration of which varied with the drying method. Colonies grown from platings of the suspensions were screened for antibiotic resistance. The suspen-

sions were in the mean time frozen and stored at $-20°$ C. to await drying. If the screening indicated satisfactory proportions of resistant and sensitive cells the suspension was considered suitable for drying. Suspensions showing between 10 and 90 % of resistant organisms were regarded as ideal for the purpose. The frozen suspensions were usually stored for no more than 24 hr. before being thawed and dried. Preliminary experiments were invariably necessary to determine the degree of instability associated with the organisms concerned and occasionally several attempts were necessary before a suitable suspension was obtained. Growth at 43·5° C. was used in one experiment (Table 2) to obtain penicillin-sensitive variants in a strain which also yielded linked kanamycin- and lincomycin-sensitive variants (Annear & Grubb, 1972*b*).

Screening for antibiotic resistance

For most of the work screening colonies for antibiotic resistance was done by multipoint replication (Annear & Grubb, 1972*a*). In one experiment where the resistant cells were only a small fraction ($< 1/1000$) of the total, their incidence was determined by surface counts on antibiotic plates. Screening of desiccates was always done on colonies which developed directly from freshly rehydrated cells.

Viable counts

In some experiments viable counts on blood agar plates were made to assess losses due to drying and storage. In other experiments only rough assessments of viability were made, by noting the density of growth on plates inoculated with heavy suspensions of either undried cells or dried cells after rehydration.

Drying methods

General. Several methods of drying were used and each involved drying suspensions from the liquid state *in vacuo* on a manifold over P_2O_5 (Annear, 1962). The suspensions were dried on various surfaces from which they could be readily recovered by gentle agitation in broth.

Method A. The organisms were suspended in 5 % sodium glutamate and single drops were dried on cellulose fibres held in small tubes (Annear, 1962). The desiccates were sealed *in vacuo* and stored at room temperature.

Methods B and C. These methods are based to some extent upon previous work (Polding, 1943; Lange & Boyd, 1968; Grivell & Jackson, 1969). Bacterial suspensions were dried on ceramic insulating beads and stored either in air or *in vacuo*. The methods are illustrated in Fig. 1. Ampoules containing approximately 50 beads (2×1 mm) were prepared and inoculated with three drops of bacteria suspended in 5 % glutamate. The inoculum was distributed over the beads by gentle shaking. The cotton-wool plug was pushed firmly into the constriction and the ampoule was dried on the manifold for 24 hr. During the first 15 min. of drying the ampoule was tapped occasionally to prevent beads sticking to each other and to the glass surfaces. The beads were then stored in two ways. For method B they were stored in small screw-capped containers in air with one or two pieces of silica gel previously sterilized by dry heat. The vessels were sealed as an added security against leakage

Table 1. *Preservation in dried cultures of unstable and linked resistance to kanamycin and lincomycin in* Staphylococcus aureus (*M* 4)

		% resistant		
			After drying	
Drying method	Storage	Before drying	Immediately	After storage
A	4 years at 25° C.	40	36	46
B	3 years at 4° C.	28	20	18
C	3 years at 25° C.	80	76	84

All cultures showed confluent growth on direct plating.

Table 2. *Preservation in dried cultures of penicillin resistance and linked kanamycin and lincomycin resistance in* Staphylococcus aureus (*M* 4) (*drying method D*)

		% resistant	
Stage of examination	Viable cell recovery (%)	Penicillin	Kanamycin and Lincomycin
Before drying	—	14	0·00021
After drying	90	20	0·00025
After storage for 4 years at 25° C.	85	16	0·00032

and were stored at 4° C. In earlier experiments, $\frac{1}{4}$ oz. glass bottles were used, but currently polypropylene tubes (Nunc No. 1078) are employed. These tubes were developed for storage of biological materials in liquid nitrogen and have proved extremely satisfactory for the purpose described here.

For method C a few beads were transferred to small tubes which were then constricted. The beads were dried for a further period of 1–2 hr. on the manifold, sealed *in vacuo* and stored at room temperature.

Method D. In this method, single drops of bacterial suspension in 10 % glutamate were dried on tufts of quartz fibres (Annear, 1964). The ampoules were sealed *in vacuo* and stored at room temperature.

Method E. Three-drop volumes of suspensions in 20 % glutamate were dried as foams (Annear, 1970). Small tubes of moist P_2O_5 (Annear, 1971) were included in the ampoules before they were sealed. Sealing was carried out in air and the desiccates were stored at room temperature. Some sealed desiccates were subjected to heating at 100° C. for 24 hr.

RESULTS AND DISCUSSION

In all experiments (Tables 1–3) high recoveries of viable organisms were obtained after drying and after storage at 25° and 4° C. Also, the screenings showed that these treatments did not modify the fractions resistant to the antibiotics concerned. The

Table 3. *Preservation in dried cultures of unstable and linked antibiotic resistance in* Staphylococcus aureus (*drying method E*)

Unstable resistance	Kanamycin and lincomycin	Kanamycin and streptomycin	Kanamycin and penicillin
Before drying			
Antibiotic resistant (%)	30	52	44
After drying			
Immediately			
Viable cell recovery (%)	85	60	90
Antibiotic-resistant (%)	40	48	40
24 hr. at 100° C.			
Viable cell recovery (%)	0·0015	0·0022	0·0034
Antibiotic-resistant (%)	28	56	40
2 years at 25° C.			
Viable cell recovery (%)	50	50	85
Antibiotic-resistant (%)	32	62	52

heating of desiccates at 100° C. in air in the presence of P_2O_5 although reducing the number of viable cells had little effect on the proportion of antibiotic-resistant organisms. Such a treatment is of prognostic value as it may accelerate some of the events likely to occur during long-term storage. Of particular interest are the results in the experiment (Table 2) in which the suspension dried had been derived from growth at high temperature and the two groups of variants had a widely separated incidence. Again the ratio of resistant to sensitive cells for each group was maintained after drying and storage.

The results presented here are representative of a much larger body of unpublished data. As well as results from organisms with linked antibiotic resistance, data from other strains in which the resistance was unlinked were also obtained. The antibiotics concerned were those mentioned in this paper and also tetracycline.

With some experiments, faulty sealing has led to moisture absorption by the desiccate as indicated by colour change of the silica gel. In such desiccates, although the viable cell content was much reduced the fraction of resistant cells invariably remained unchanged.

In comparing the various methods of drying investigated here, both the fundamental and applied aspects of the work should be considered. For example, the sealing of desiccates in the presence of P_2O_5 provides data from desiccates stored under defined conditions but is not a procedure with practical appeal. Where drying of cultures for no particular immediate purpose is undertaken, method A has remained the routine. It has proved highly reliable since its adoption some 10 years ago (Annear, 1962). There has, however, been an increasing trend towards the use of methods B and C which may be conveniently carried out together. The storage of beads in air (method B) has great practical appeal and has undoubtedly stimulated and accelerated studies of antibiotic instability in this laboratory. The ready availability of a large number of standard inocula in one small readily accessible vessel is of obvious value. However, until more quantitative evidence is available, it is considered wise to maintain some of the beads *in vacuo* (method C).

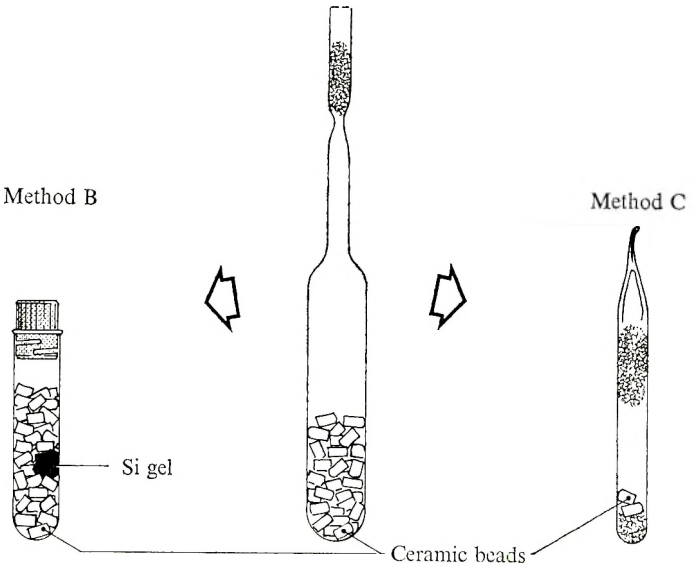

Fig. 1. Preparation of desiccates on ceramic beads.

As yet, no reliable evidence has been obtained in this laboratory for drying organisms other than staphylococci by method B, although several workers already cited have enthusiastically recommended the method for a number of other organisms.

The temperature of 4° C. has been arbitrarily adopted for storage as likely to give better survival rates than room temperature. The silica gel in the sealed desiccate has been included not for the purpose of achieving or maintaining dryness but as an indicator of effective sealing. The final equilibrium humidity level is not known or controlled with any precision but is obviously reproducible within reasonable limits.

While it is usual to preserve cultures being used in genetic projects as a precaution against loss, contamination or change, their preservation at various stages of such experimentation and as an integral part of it does not appear to be widely practised. The advantage of such a practice is that it enables experiments to be interrupted or to be reinvestigated at definite points. It also relieves the pressure for immediate investigation where rapid changes are occurring and enables the investigator to plan his work more conveniently and under less duress. More specifically, it has been found that one of the advantages of 'characterized' desiccates in studies of unstable antibiotic resistance has been their ready availability for testing for additional linkages to those already recognized.

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Observations on the microbiological flora of canned Parma ham

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SUMMARY

A significant proportion of a consignment of branded Parma Ham was found to have 'blown' tins; the associated bacterial flora was therefore investigated. No *Salmonella* or *Shigella* were found. *Clostridium welchii* type A and *Clostridium bifermentans* were isolated in moderate numbers only from enrichment cultures of the ham. *Staphylococcus* spp. and coryneform bacteria were obtained from all tins and Group D streptococci were present in a few. No food-poisoning cases were associated with this ham.

INTRODUCTION

Ham of the Italian type is a popular food in Britain and with entry into the European Economic Community there is every likelihood that its consumption will increase.

In the Parma province alone production reaches 35,000 metric tons per year and so far there have been few bacteriological studies of the food. Giolitti, Cantoni, Bianchi & Penon (1971) found that in lean hams the microflora counts remained at low figures during the curing process; slightly higher figures were found in ham fat. They concluded that in the ripening process the microflora exerted only a minor influence and that in normal and sour hams bacteriological spoilage appeared not to play a significant role.

The product which we examined was a sliced, salt-cured ham from the Parma region which had not been subjected to any cooking process before canning. A W.H.O. expert committee (1968) point out that with raw ham the major risk is the development of *Clostridium botulinum* infection.

Matveev, Nefedjeva, Bulatova & Sokolov (1967) in the U.S.S.R. indicated that during recent years a serious danger in connexion with botulism appeared to be various home-processed pork products, and in fact pork products were the third most common cause of botulism in Russia; *Cl. botulinum* type B was most commonly isolated from pork. There are no records of botulism in Canada from sliced, commercially packed meats (Thatcher, Erdman & Pontefract, 1967) although Reed, Butas & Gall (1965) described a small outbreak caused by canned liver paste.

Because of the possibility of pathogenic bacteria, particularly anaerobes, being responsible for the 'blowing' of the tins in the present study, an attempt was made

using routine laboratory methods to isolate the responsible organisms. In no way could this be considered a research investigation; it was an endeavour to solve an urgent public health problem with limited facilities and resources.

MATERIALS AND METHODS

The number of 6 oz. cans examined at the warehouse by the Public Health Inspector totalled 1,008; 218 of these tins were found to be 'blown'. Similarly, out of 144 12 oz. tins, 105 were 'blown'. The consignment was stored at between 0 and 2° C. in the warehouse but had been kept at 'room temperature' for some days before transference to the cold room. Twenty-three of the 6 oz. cans were collected by the Public Health Inspector for laboratory examination.

The cans were made from coated, pressed, mild steel with a crimped and soldered double seamed joint and were of the 'round flat' type, the exhaust hole being plugged with solder after processing. These were checked for defects but all were sound, none showing any evidence of 'blowing'. Storage instructions on the lid read 'Keep in a cool place'.

Three of the tins were examined for bacteriological culture direct from the warehouse. The remaining 20 were kept at three different temperatures for 2 days before bacteriological examination. Five were kept at 4°, 10 at 22° and 5 at 30° C. The tins were washed with soap and water and treated with 70% alcohol before opening (Cruickshank, 1965). Duplicate 1 g. samples were taken from each tin using a sterile cork-borer. The samples were homogenized in 50 ml. (sample 1) and in 5 ml. (sample 2) of quarter-strength Ringer's solution.

Tenfold dilutions were made from sample 1 and pour plates made from this using Dextrose Tryptone Agar (Oxoid) (D.T.A.) and Plate Count Agar (Oxoid) (P.C.A.). The D.T.A. plates were incubated at 37° and 55° C. The P.C.A. plates were incubated at 22° and 37° C. Using the same dilution and a modified Miles & Misra (1938) technique (Sutton, Ghosh & Hobbs, 1971) plate counts were made on duplicate blood agar (B.A.) plates and incubated at 37° and 22° C. Special media for the selective isolation of the aerobic flora were not used. Sample 2 was examined by spreading 0·5 ml. on the surface of duplicate blood-agar plates which were incubated at 37° C. under anaerobic conditions for 48 hr. A total anaerobic count was made by adding together the colony counts from each plate. Two ml. of sample 2 was inoculated into a tube of cooked-meat broth and heated to 100° C. for 30 min., incubated at 37° C. overnight and subcultured on Neomycin (Upjohn) blood agar plates for the presence of heat-resistant spores. Further ham from each tin was inoculated into Robertson's cooked-meat broth, McConkey broth and Selenite F enrichment medium and incubated for 48 hr. at 37° C. and the subculture examined after a further 48 hr. at the same temperature.

On completion of the bacteriological studies four of the tins were examined for water content and NaCl concentration; the pH was estimated on 11 tins; sodium nitrite and nitrate concentrations were also determined on the meat.

Table 1. *Total viable bacterial plate count per gram of ham*

Media and incubation temperature	Storage temperature for 48 hr. before opening															
	4° C.						22° C.						37° C.			
D.T.A., 37° C.	1	1·6	6	93			11	1·2			16	72				
	2	4·6	7	86			12	13			17	22				
	3	150	8	19			13	1			18	6·4				
	4	48	9	26			14	26			19	1·5				
	5	19	10	25			15	19			20	120				
		× 10^3		× 10^3				× 10^3				× 10^3				
D.T.A., 55° C.	Nil						Nil				Nil					
B.A./P.C.A., 37° C.	1	930	6	1250			11	1040			16	400				
	2	990	7	540			12	750			17	4500				
	3	680	8	1030			13	1520			18	480				
	4	1280	9	1980			14	2050			19	440				
	5	450	10	1150			15	1560			20	600				
		× 10^3		× 10^3				× 10^3				× 10^3				
B.A./P.C.A., 22° C.	1	144	6	900			11	670			16	120				
	2	230	7	430			12	670			17	510				
	3	290	8	740			13	670			18	110				
	4	350	9	730			14	980			19	20				
	5	100	10	800			15	700			20	280				
		× 10^3		× 10^3				× 10^3				× 10^3				

RESULTS

The degree of firmness of the tins was assessed after 48 hr. storage at different temperatures. All the tins stored at 30° C. felt soft to finger and thumb pressure. Several of those kept at 22° C. were likewise soft, and all those kept at 4° C. felt firm. Of the 12 soft tins, 11 produced cultures of anaerobes; from 2 of the 8 firm tins similar organisms were isolated. In each case *Cl. welchii* was isolated from a mixed anaerobic culture.

The average wet-weight NaCl content of the ham was 3·93 % and the moisture content 46·94 %, these figures giving a concentration of 8·4 g. salt/100 g water. The average pH of the tins examined was 5·95; the sodium nitrate concentration was less than 1 p.p.m. whilst the sodium nitrite concentration was 2·8 p.p.m.

Variation in storage temperatures (range 4–30° C.) for 48 hr. before sampling the tins appeared not to affect the concentration and the distribution of the bacterial content (Table 1).

Comparison was made with the total viable plate counts obtained from the blood agar plates and the pour plate method using Plate Count agar. The average of the counts was taken and is given as B.A./P.C.A. in Table 1.

Staphylococci accounted for 70 % of the total aerobic plate count, assessed from the blood agar plate. Packet-forming Gram-positive cocci with a typical colonial appearance and a diameter of not less than 0·5 mm. were selected for further study and classified as staphylococci according to Cowan & Steel (1966). The organisms were coagulase-negative and fermented glucose and mannitol. Enterotoxins A, B and C were not produced by the three cultures so examined.

Colonies having the morphological characteristics of *Streptococcus faecalis* were identified as Lancefield Group D streptococci and were isolated from seven of the

tins; this organism grew well on 10% bile salt medium, fermented sorbitol and was grouped using the formamide extraction method of Fuller (1938). An organism with similar morphological appearances was identified as a Group C streptococcus using the same technique. This organism was isolated from only one of the tins and only in small numbers.

Small Gram-positive rods growing well aerobically were identified as coryneform bacteria. These organisms were present in all the tins and were the second largest group isolated. The presence of lactobacilli could not be demonstrated under aerobic or microaerophilic conditions on routine culture media.

A heat-resistant *Cl. welchii* type A was isolated from two of the tins. A proteolytic Gram-positive rod with oval subterminal spores was obtained from 13 of the tins. The results from a number of biochemical tests indicated that this organism was a *Cl. bifermentans*. This was confirmed by the Food Hygiene Laboratory at Colindale, where culture filtrates from this organism did not kill mice. In each case the anaerobes were isolated on subculture from cooked-meat medium. No other anaerobes were isolated.

Because of the local lack of suitable animals, adequate studies of possible *Cl. botulinum* toxin and spores were not feasible and the isolation of vegetative forms only was attempted.

DISCUSSION

Food-poisoning outbreaks due to staphylococcus enterotoxin and to *Cl. welchii* remain common (Thatcher, 1963) and deaths from botulism were recorded by both Dauer and Dolman in 1961.

A significant proportion of Parma ham tins were found to be 'blown' but the meat showed no evidence of serious spoilage or putrefaction; storage at 4° C. appeared to reduce the number of 'blown' tins; *Cl. botulinum* was never isolated.

The average pH of a small number of cans was 5·95; *Cl. botulinum* growth can occur if the pH is above 5·0 and the relative humidity above 94% (W.H.O. Committee, 1968); on the other hand, it cannot germinate and grow in a strongly acidic environment, reduction of the pH could thus possibly be an extra safety factor. The W.H.O. Committee (1968) state that the limiting salt concentration for types A and B *Cl. botulinum* is 8–9%; this percentage being based on the concentration of salt in the aqueous phase of the food. Moreover Spencer (1967) found that in cured meats 14–15 p.p.m. nitrite allied with 4·9 g salt/100 g water at a pH of 6·4 had an inhibitory effect on type A *Cl. botulinum* both in growth and toxin production. In comparison with these results, the Parma ham figures suggest that the sodium nitrite (2·8 p.p.m.) and sodium nitrate (less that 1 p.p.m.) are low but that the sodium chloride concentration (8·4 g/100 g water) is probably acceptable in a food where safety is partially dependent on the concentration of curing salts to prevent outgrowth of the bacteria.

Spencer (1967), however, found that the concentration of salts necessary to prevent growth of *Cl. botulinum* depends on the number of vegetative cells present, and as these are usually sparse in food the concentration of curing ingredients, although low, might well provide acceptable safety levels.

Cl. bifermentans was found in 13 of 20 tins, particularly those stored at 22° C. and over; this is not an organism likely to cause food-poisoning although it can produce an exotoxin; its role is more that of an indicator organism than a pathogen. *Cl. welchii* was isolated in moderate numbers from 2 of 20 tins in mixed culture with other anaerobes. Sutton *et al.* (1971), however, point out that large quantities of *Cl. welchii* (millions/g) are necessary to cause clinical food poisoning; Hobbs & Gilbert (1970) confirm this. Giolitti *et al.* (1971) isolated no anaerobes in their investigation of raw ham although the meat was 'untinned' salt cured ham, unlike the tinned slices in the present study.

The curing of the hams is done by external dry rubbing of salt followed by ripening for 6–12 months or longer; the exact preparatory process is a trade secret. *Staphylococcus*, the only other potential pathogen isolated, grows well at a pH above 4·5 and in a salty environment, both these conditions being fulfilled in this ham. No large numbers of staphylococci were isolated and no clinical symptoms would occur after eating this meat.

The cause of the 'blown' containers is difficult to assess; bacterial gas production is likely although putrefaction was not present. Both *Cl. welchii* and *Cl. bifermentans* have saccharolytic and proteolytic properties. Enzymic hydrolysis, suggested by Giolitti *et al.* (1971), may be a factor and expansion of trapped air within the cans may be another possible cause. Whether keeping this consignment at 'room temperature' for some days before cold room storage caused or increased the number of 'blown' tins is not known but could be of some significance in view of the experimental results. Neither the vacuum nor the head space gases in the cans were determined.

The bacteria present in the raw ham could have been introduced into the meat during the slicing or canning procedures, although lactobacilli have been found in pig carcasess and at all stages in the production of bacon (Riddle & Spencer, 1966; Kitchell & Ingram, 1967; Riddle, 1968). Mol *et al.* (1971), in a careful study of vacuum-packed cooked meats, state that unclassified streptobacteria eventually spoil the food because they grow well at low temperatures under anaerobic conditions and that variations in salt, pH and nitrite do not apparently curb their growth; similar organisms have also been isolated from raw cured meats (Cavett, 1962, 1963) in addition to lactic acid bacteria and micrococci (Kitchell & Ingram, 1963). Coryneform bacteria and *Staphylococcus* spp. were found in all the tins examined in this study and may play a part in eventual spoilage of meat, as may the streptococci Group D; they do not, however, produce food-poisoning in humans.

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