J. Hyg., Camb. (1963), **61**, 265
Printed in Great Britain

The effect of desiccation on the viability of *Staphylococcus aureus*

By PHYLLIS M. ROUNTREE
Fairfax Institute of Pathology, Royal Prince Alfred Hospital, Sydney

(*Received* 16 *February* 1963)

INTRODUCTION

People infected with *Staphylococcus aureus* or carrying this organism can contaminate their bedding and surroundings (Hare & Cooke, 1961; Rountree & Beard, 1962) and pathogenic staphylococci are frequently isolated from air samples collected in hospitals. Lidwell, Noble & Dolphin (1959) found these air-borne staphylococci to be carried on particles of small size, the majority being less than 18 μ. It is presumed that these staphylococci in the environment of patients are in the dried state.

Do these dried organisms retain their ability to infect fresh hosts? And if so, for how long after they have been shed? These are questions of some importance when considering measures for the control of staphylococcal sepsis in hospitals. Some circumstantial evidence (Rountree & Beard, 1962) suggests that cocci deposited on ward blankets by a patient can colonize the nose of the next occupant of the contaminated bed.

Lidwell & Lowbury (1950) examined the death-rates of *Staph. aureus* present in samples of dust collected in three hospitals. The death-rate per day varied with the relative humidity at which the dusts were stored, ranging from 0·030 in a dry atmosphere to 0·53 at 84% relative humidity.

Maltman, Orr & Hinton (1960) studied the effect of drying on glass at room temperature on the survival of the Wood 46 strain of *Staph. aureus*. They found that the viable count was reduced approximately 90% after 6 days. The dried organisms also showed a prolongation of the lag phase which increased with the time of storage, an increase in the time taken to clot plasma and a diminished survival rate in human serum. Further observations (Hinton, Maltman & Orr, 1960) indicated that these dried cells had lost much of their virulence for mice by the intramuscular, intravenous and intracerebral routes.

Wood 46 was isolated many years ago (Burnet, 1929) and its behaviour may not be identical with that of strains of more recent origin. Furthermore, organisms dried on glass may differ from those dried on textiles. The question of the effect of desiccation on the viability of staphylococci has therefore been further studied in a system which attempts to simulate the situation when organisms are shed from the body on to textiles.

METHODS

The strain to be dried was inoculated into broth from an overnight culture and was grown at 37° C. for 3–4 hr. until it contained $1–2 \times 10^8$ cells/ml. Inocula of 0·1 ml. were then deposited on sterile pieces of textile approximately 10 mm. square into which they soaked rapidly. Ten to twenty squares were prepared for each strain and were stored for drying in sterile Petri dishes in a cupboard at room temperature. The temperature and relative humidity in the cupboard were observed daily; the temperature varied from 66 to 70° F., but on a few occasions rose to 80° F. and the R.H. ranged from 42 to 50 %.

For most of the observations, cotton lint of the type used for covering surgical wounds was employed. A few observations were made with woollen blanket and cellular cotton ('Osman') blanket. All textiles were washed and autoclaved before use.

Counting. Viable counts were made by placing a square in 5 ml. of broth in a 1 oz. screw-capped universal container. This was shaken for 2 min. and appropriate tenfold dilutions then made in broth held in an ice-bath. Four or more samples of 0·01 ml. (measured with a calibrated pasteur pipette) were placed on quadrants of blood agar plates and spread with a sterile glass rod until the liquid had adsorbed into the medium. Colonies were counted after overnight incubation at 37° C.

Preliminary experiments showed that all the colony-forming units deposited on a square of textile could be recovered by this method. For example, a broth culture of PS 80 gave a count of $1·6 \times 10^8$ colony-forming units/ml. just before depositing 0·1 ml. volumes on the lint, and $1·5 \times 10^7$ colonies were grown from the shaken material. Similarly, a culture of Wood 46 with a count of $1·05 \times 10^8$/ml. yielded $1·1 \times 10^7$ colonies per square. For reasons of convenience the counts will be expressed as cocci/square without applying any corrections for pairs or larger groups of cocci.

Death-rates. K, the death-rate per day, was calculated with the following formula:

$$K = 2·3 \frac{B_0 - B_t}{t},$$

where t = time of drying in days, $B_0 = \log_{10}$ count at time 0, and $B_t = \log_{10}$ count at time t.

Strains of staphylococci. The strains examined came from a variety of sources. Two strains, Wood 46 and Bundaberg, isolated many years previously, had been maintained in culture media before reaching this laboratory where they have been dried. Others had been kept as dried cultures. The majority of strains were examined within one subculture of their isolation from air samples or patients. All strains were phage typed using the basic international set of typing phages (Blair & Williams, 1961) plus two phages of local interest, 31 B and 47 D.

RESULTS

Multiplication of the cocci on textiles. Cultures deposited on the squares and stored in the cupboard appeared to be dry within 24 hr. Counting showed that multiplication had taken place during this period. Squares on which 1×10^7 cocci had been placed gave a count of 1×10^8 or more on the next day. Accordingly, the counts on day 1 and not those of day 0 were used as a base-line. It cannot be excluded that deaths occurred in these first hours of drying but, in view of the slow fall in counts of most of the strains, this is unlikely to be of any significance.

Comparison of Wood 46 *with other strains.* Fig. 1 illustrates the behaviour over a period of 3 months of Wood 46, of PS 80 (the propagating strain of phage 80), of a strain of phage group III showing multiple antibiotic resistance, and of a strain of phage group II. The first three strains were set up on the same day and the group II strain 4 weeks later.

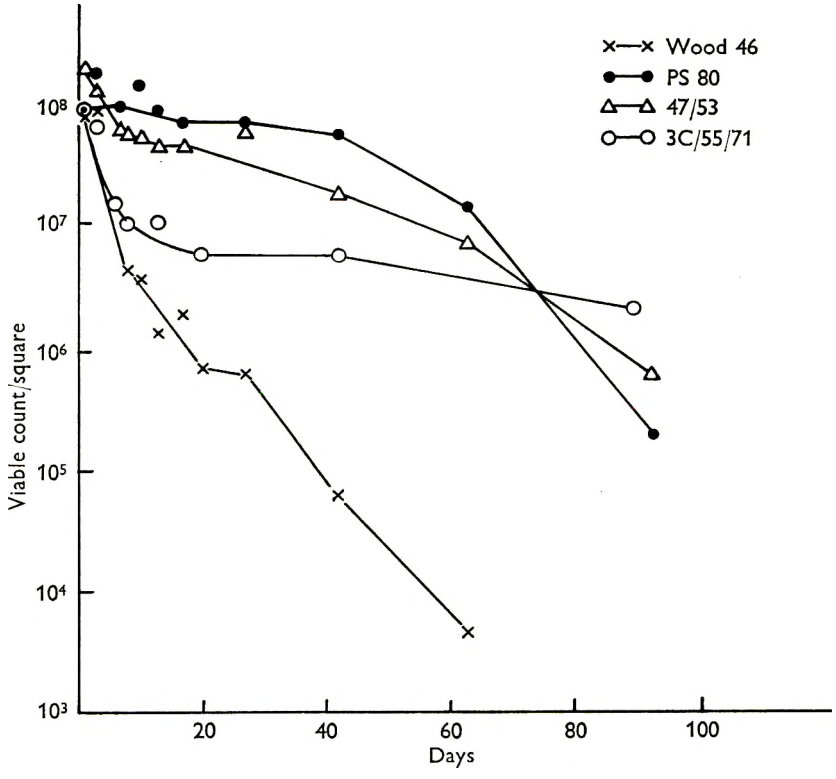


Fig. 1. Loss of viability of four strains of *Staphylococcus aureus* dried on cotton lint.

The count of Wood 46 fell by 2 logs during the first 2 weeks of drying; thereafter, the decline in count was more gradual to the 28th day but at the end of 93 days no viable cells could be recovered. In contrast to this, strain PS 80 showed no significant loss of viability at the 14th day and very little loss between the 14th and 42nd days. The survival curves of the two other strains resembled each other, showing initial loss of viability over the first 14–18 days and then a flattening of the curves. In contrast to Wood 46, all three strains had viable cells at the end of 3 months desiccation.

The question of whether the morphological clumping of the cocci might have any effect on the shape of the curves, particularly during the early period of drying, was considered. PS 80, which showed no apparent loss of viability during the first 14 days, might, in fact, be losing viable cells from pairs or clumps which would still register as a viable unit. Extrapolation of the curve backwards from its slope of the 14th to 42nd day did not however indicate that the count at day 1 was subject to an error of any great magnitude, and it was concluded that there was no significant loss of undetected viability, during the first period of drying.

Loss of viability by various strains. A total of thirty-eight strains was examined for their loss of viability, when dried on lint.

The strains could be divided into two categories, those whose viable count fell from the beginning of the drying period and those that showed no significant loss of viability until after the 14th day or later.

Table 1 gives details of the strains that fell into the first category. The death-rates varied from 0·031 to 0·278/day. It was notable that the two old laboratory strains, Bundaberg and Wood 46, had the highest values for K.

Table 1. *Death-rates of strains of* Staphylococcus aureus *showing loss of viability within* 15 *days of drying*

Strain no.	Phage group	Phage pattern	Source	Log loss at day 15	Death-rate, K/day at day 15
Bundaberg	I	52/52A/80/81		1·1092	0·17
Haines	I	29/52/52A/79/80	Nasal swab	0·2876	0·043
M4	I	52/52A/80/81	Air sample	0·2382	0·039
3085	I	29	Nasal swab	0·3736	0·057
3095	I	52	Nasal swab	0·2001	0·031
3100	I	29/52	Nasal swab	0·3688	0·057
136	II	3C/55/71	Air sample	0·9208	0·15
672	II	3A/3B/3C	Infected ulcer	0·9488	0·145
AR	II	55/71	Air sample	0·7090	0·109
3028	II	55/71	Surgical wound	0·4327	0·066
2807	II	3B/3C/55/71	Osteomyelitis	0·4463	0·068
2812	III	7/31B/47D	Abscess	0·4994	0·077
1002	III	53	Empyema	0·6632	0·101
2502	III	53	Blood culture	0·6637	0·101
SS6/1	N.C.	Groups I–III	Air sample	0·4046	0·062
Wood 46	—	N.T.	—	1·8110	0·278
814	—	N.T.	Nasal swab	1·000	0·153
815	—	N.T.	Nasal swab	0·3553	0·055
817	—	N.T.	Surgical wound	0·7959	0·122

For those strains that showed no significant loss during the first part of the drying period, the death-rates were calculated over that period of time when their counts were falling. Details of these strains are shown in Table 2. The death-rates/day ranged from 0·018 to 0·137.

Effect of sunlight on the death-rate. A culture of PS 80 was dried on squares of lint, half of which were stored in the dark and the other half exposed to the effect

Table 2. *Death-rates at later times of strains of* Staphylococcus aureus *showing no loss of viability during first* 15 *days of drying*

Strain no.	Phage pattern	Source	Days of drying	Log loss	Death-rate, K/day
PS 80	80/81	Epidemic of newborn, Sydney (1953)	13–63	0·7696	0·035
PS 80/1 *a*	52/52A/80/81	Mutant of PS 80	15–50	1·2083	0·079
PS 80 (*a*)	52/52A/80/81	'Converted' PS 80	21–65	0·6989	0·038
PS 80 (*n*)	52/52A/80/81	'Converted' PS 80	15–65	1·1498	0·055
U 9	80/81	Epidemic of newborn, U.S.A. (1954)	15–50	1·6173	0·106
9684	52/52A/80/81	Epidemic of newborn, Victoria (1954)	15–50	2·0792	0·137
Allen	52/52A/80	Epidemic, Atlanta, U.S.A.	22–50	1·6600	0·136
Bellville	52/52A/80	Epidemic, Atlanta, U.S.A.	15–50	1·7233	0·113
594B	52/52A/80/81	Empyema	29–65	0·8171	0·052
3082	52A/79	Nasal swab	15–28	0·4102	0·072
	52A/79		28–64	1·2323	0·080
3023	6/54	Infected surgical wound	15–29	0·5376	0·088
820	7/47D	Sputum	15–38	1·3310	0·133
	7/47D		38–91	1·9313	0·084
2994	42E	Infected surgical wound	29–92	3·2821	0·120
PP 7/1	42E	Air sample	36–84	0·5878	0·029
1009	47	Infected surgical wound	15–65	1·6990	0·078
8503	53	Cross-infected empyema	15–66	1·3388	0·060
2825	77	Sputum	15–38	0·1841	0·018
3027	77	Infected ulcer	15–92	3·6484	0·108
670	42D	Nasal swab	15–64	0·8921	0·042

Table 3. *Loss of viability of two strains of* Staphylococcus aureus *dried on cotton cellular blanket and on woollen blanket*

	Cotton			Wool		
Strain no.	Day	Log loss	Death-rate, K/day	Day	Log loss	Death-rate, K/day
PS 80	7	0·7634	0·25	7	0·1677	0·055
	15	1·4847	0·227	15	0·2156	0·033
	21	1·7392	0·190	—	—	—
	30	2·5873	0·198	28	0·3406	0·028
	51	4·6700	0·210	51	1·1577	0·052
8503	7	0·2853	0·093	7	0·0969	0·031
	15	0·6072	0·093	15	0·1426	0·022
	21	0·7633	0·084	21	0·2218	0·024
	28	1·2304	0·104	28	0·1938	0·016
	51	2·1512	0·097	51	1·0555	0·048

of indirect light on a ledge behind the laboratory window. Counting over a period of 21 days showed that the death-rate/day in the light was 0·432 compared with 0·029 in the dark.

Effect of different textiles on the death-rate. Two strains were examined for their death-rates on cotton and on woollen blanket. The strains, PS 80 and 8503, were set up on both textiles on the same day, so that conditions during the drying period were identical. The counts (Table 3) showed that survival on the woollen material was prolonged for both strains, little or no loss occurring until the end of 28 days. (The difference in counts before this are within the range of experimental error.) By contrast, on the cotton blanket, PS 80 died off quickly, the mean value of K being 0·21. Strain 8503 was more resistant to drying on this material, the mean value of K being 0·095 . It was concluded that the nature of the textile on which the staphylococci were dried had a considerable effect on their survival.

Relationship of survival on drying to mercury resistance

Moore (1960) reported that 'epidemic' strains of staphylococci were more resistant to mercury salts than non-epidemic strains. For example, in phage group I, practically all strains lysed by phage 80, and in group III, a large proportion of those frequently associated with epidemics of wound infection, were mercury resistant. The mechanism of the mercury resistance is unknown but the possibility that the strains showing no loss of viability in the early part of the drying period might also be mercury resistant was discussed with Dr Moore. Some of the strains used in the drying experiments had been tested for mercury resistance and, although strains lysed by phage 80 were also mercury resistant and resistant to drying, many exceptions to this association were found. For example, Wood 46 was mercury resistant.

The death-rates of three related strains received from Dr Moore were determined after drying on lint. Strain 75 AY was penicillin and Hg resistant, and the death-rate at 13 days was $K = 0{\cdot}101$. A penicillin-resistant, Hg sensitive strain, 75B, also had a value for K of 0·101 while a Hg-sensitive, penicillin-sensitive strain, 75 C, gave $K = 0{\cdot}130$.

DISCUSSION

The conditions in which the death-rates of these staphylococci were measured were arranged to be comparable to those in hospital wards in this climate. The work of Lidwell & Lowbury (1950) on the death-rates of staphylococci in stored hospital dust showed that the relative humidity had an important influence on the survival of the organisms. The present experiments were carried out for the most part over a fairly narrow range of temperatures and relative humidities. It was noted, however, that on several occasions during the summer when temperature and humidity rose there was an accelerated death-rate in the strains then being studied. Such results have been excluded from this report. It would be desirable for this study to be repeated under controlled climatic conditions.

The strains that showed no significant loss of viability for the first fortnight of storage are of particular interest. Most were strains isolated from epidemics of infection in this hospital or elsewhere, or were of phage types implicated in such infection in other parts of the world, e.g. 52A/79. Mutants of PS 80 whose phage pattern had been altered by loss of their defective prophage (Rountree & Asheshov, 1961) retained the resistance to drying of the original wild type. Among the group III strains were two, viz. 1009 and 8503, showing resistance to many antibiotics and representative of strains that have caused numerous infections in this hospital. These strains showed resistance to drying but, on the other hand, two strains of similar character, 1002 and 2502, did show significant loss of viability in 15 days. Evidently, the correlation between resistance to drying and ability to cause epidemics is not complete.

All the strains of group II that were tested died off relatively rapidly. In general, strains of this group are rarely implicated nowadays in hospital epidemics.

The two strains with the highest death-rates were the old laboratory strains, Wood 46 and Bundaberg. Whether these death-rates are the result of their maintenance in laboratory media for many years cannot now be determined. On the basis of their results with Wood 46, Hinton *et al.* (1960) suggested that staphylococci shed from the body and surviving in the hospital environment had suffered damage which decreased their potential for infection. The present results indicate that Wood 46 behaves differently from more recently isolated strains and cannot be regarded as a typical 'hospital' strain. From the data in Table 2 of the paper by Maltman *et al.* (1960), K for Wood 46 dried on glass was calculated as 0·18 at 14 days; this value was not as high as that obtained in the present investigation with drying on lint.

The ability to survive for prolonged periods on textiles could be a character that gives a strain a selective advantage in its occupation of the ecological niche of the modern hospital in a temperate climate. The fact that the majority of strains implicated in recent hospital epidemics survived for at least a fortnight with undiminished viability might be sufficient to give them a slight selective advantage over strains that died more quickly. Their survival could allow them greater chances to come in contact with new hosts who need not necessarily develop clinical infection; establishment of the carrier state would be sufficient to perpetuate the strain. The question of whether the dried and stored staphylococci can initiate lesions is now being re-examined.

Recent discussion on the respective merits of various types of textile used in the composition of hospital blankets have been concerned chiefly with their ease of laundering. The present studies indicated that on material with an open weave such as cellular cotton, death-rates of staphylococci were faster than on woollen blanket material. This might be an additional factor to be considered in choosing which type of blanket to use in hospitals.

SUMMARY

The death-rates have been determined of strains of *Staphylococcus arueus* dried on lint and other textiles and stored at room temperature in the dark.

Two categories of strains were distinguished, those that showed no significant loss of viability after 15 days storage and those whose death commenced from the beginning of the drying period.

There was some degree of correlation between survival for 15 days and implication in epidemics of hospital infection.

REFERENCES

BLAIR, J. E. & WILLIAMS, R. E. O. (1961). Phage typing of staphylococci. *Bull. World Hlth Org.* **24**, 771.

BURNET, F. M. (1929). The exotoxins of *Staph. pyogenes aureus*. *J. Path. Bact.* **32**, 717.

HARE, R. & COOKE, E. M. (1961). Self-contamination of patients with staphylococcal infection. *Brit. med. J.* ii, 333.

HINTON, N. A., MALTMAN, J. R. & ORR, J. H. (1960). The effect of desiccation on the ability of *Staphylococcus pyogenes* to produce disease in mice. *Amer. J. Hyg.* **72**, 343.

LIDWELL, O. M. & LOWBURY, E. J. (1950). The survival of bacteria in dust. II. The effect of atmospheric humidity on the survival of bacteria in dust. *J. Hyg., Camb.*, **48**, 21.

LIDWELL, O. M., NOBLE, W. C. & DOLPHIN, G. W. (1959). The use of radiation to estimate the numbers of micro-organisms in air-borne particles. *J. Hyg., Camb.*, **57**, 299.

MALTMAN, J. R., ORR, J. H. & HINTON, N. A. (1960). The effect of desiccation on *Staphylococcus pyogenes* with special reference to implications concerning virulence. *Amer. J. Hyg.* **72**, 335.

MOORE, B. (1960). A new screen test and selective medium for the rapid detection of epidemic strains of *Staph. aureus*. *Lancet*, ii, 453.

ROUNTREE, P. M. & ASHESHOV, E. H. (1961). Further observations on changes in the phage-typing pattern of phage type 80/81 staphylococci. *J. gen. Microbiol.* **26**, 111.

ROUNTREE, P. M. & BEARD, M. A. (1962). Observations on the distribution of *Staphylococcus aureus* in the atmosphere of a surgical ward. *J. Hyg., Camb.*, **60**, 387.

J Hyg., Camb. (1963), **61**, 273
With 2 plates
Printed in Great Britain

The measurement of bacterial respiration on pig skin with micro-respirometers

By R. C. S. WOODROFFE
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire

(*Received* 21 *February* 1963)

INTRODUCTION

The effect of germicides on the bacterial flora of skin is usually estimated during hand-washing tests, and these techniques have been reviewed elsewhere (Hurst, Stuttard & Woodroffe, 1960). Such tests are time-consuming and require the co-operation of human volunteers. It would be possible to reduce the number of these tests if a suitable *in vitro* test was available by which germicides could be screened to discover the most potent before doing hand-washing tests. One possibility for achieving this screening is to assess the effect of germicides on some activity of the bacteria which is measurable while bacteria and germicide are together on the skin. Bacterial respiration was selected for study. Before developing a suitable test it was necessary to find an experimental system in which such respiration could be measured.

Amongst the reported methods of measuring respiration, the one most readily adaptable for the purpose of measuring bacterial respiration was that of Cruickshank (1954). Cruickshank used Differential Capillary micro-respirometers to assess the toxicity of various agents to skin respiration. He and later workers (Cruickshank, Trotter & Cooper, 1957; Lawrence & Ricketts, 1957; Lawrence, 1959*a*) used skin from the ears of guinea-pigs, which is relatively free from bacteria. The skin was suspended in a medium containing streptomycin to suppress bacterial contamination.

It would be desirable to work with human skin in the respirometer, but it was not possible to obtain this and therefore a suitable substitute was found. Skin from a number of animals was examined and pig skin was selected because the number and type of bacteria were similar to those found on human hands (Baird-Parker, 1962). These bacteria are predominantly Gram-positive cocci which are in both cases in the stratum corneum and the sweat glands.

This paper describes the modification of Cruickshank's method to enable the bacterial respiration to be measured, and discusses some of the experimental variables of the system.

MATERIALS AND METHODS

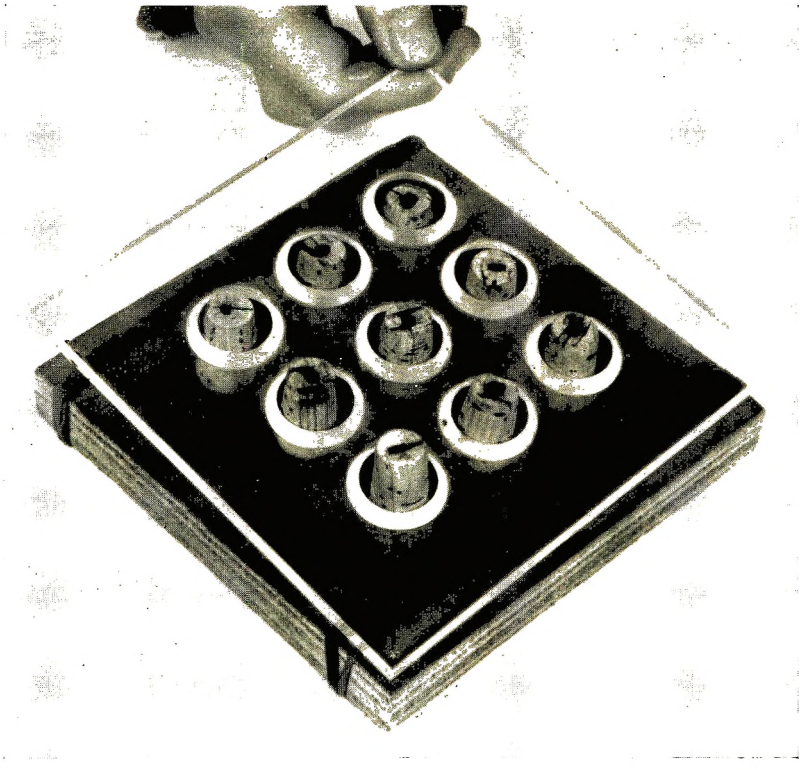
Preparation of skin. Immediately after pigs were killed trotters were cut from the fore-legs and packed in solid CO_2 for overnight transportation to the laboratory in a refrigerated van. On receipt, blood and dirt were removed under running

water using a nail brush. After drying, hair was removed with electric clippers. A strip of skin about 8 × 6 cm. was excised from the back of the pastern joint of a pig's trotter. This area was chosen for convenience and skin from other sites could probably be used. A strip of skin could be divided into 2 or 3 parts as required. Each piece of skin was laid over a cork previously mounted on a wooden block. Two wooden blocks were used, one for test skins and the other for control skins, each carrying nine corks. Strips of skin from a number of trotters could therefore be included on each block. After the skin had been placed over each cork with stratum corneum uppermost, a plastic template containing holes to coincide with the skin was pressed over the skin samples and secured to the wooden block with rubber bands. Bulges of skin, 2 cm. diameter, were thereby exposed and could be treated as required (Pl. 1).

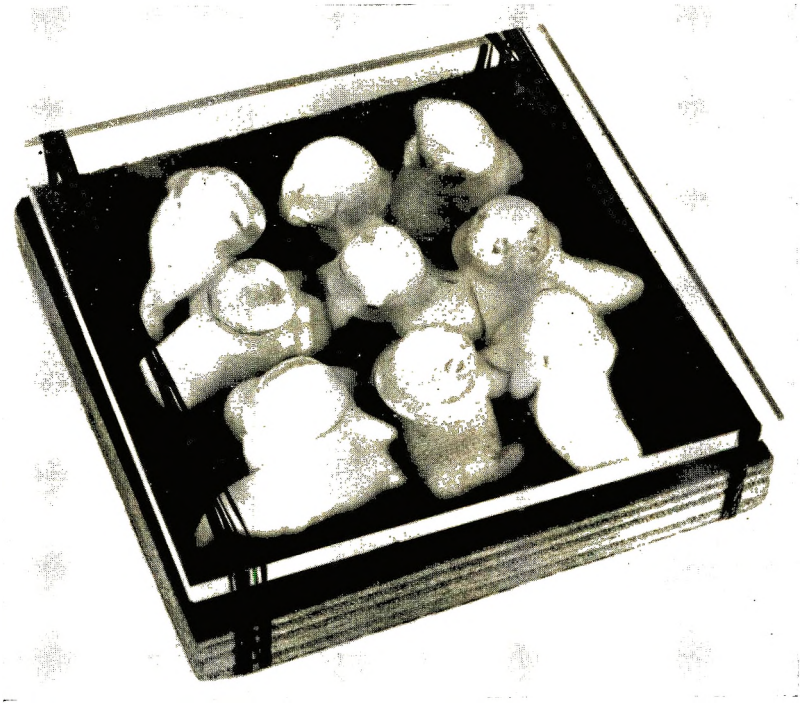
Microrespirometry. The Differential Capillary micro-respirometers designed by Cruickshank (1954) permitted continuous observations of oxygen uptake of skin and the admission of liquid to the tissue during experiments. The apparatus consisted of two nickel-plated brass blocks which were held together by bolts passing through both blocks and secured by wing-nuts. The contact surfaces of the blocks were ground flat so that when they were lightly greased and clamped together they were air-tight. In the ground face of each block two circular recesses were positioned so that, when the blocks were fitted together, identical chambers were formed.

Filter-paper disks moistened with 2·0 % potassium hydroxide to absorb carbon dioxide emitted by the skin and glass cups to carry 1·0 ml. medium with skin samples were placed in the chambers. The medium contained Krebs–Ringer buffer, glucose, serum from the same source as the skin, and streptomycin. The chambers were connected by a calibrated, volumetrically graduated capillary tube containing indicator fluid, cemented into holes which passed through the top block immediately over the chambers. Pivoted at the centre of the blocks was a rotary gassing tap consisting of a Perspex disk having a central hole flanked by two metal gassing tubes which were screwed and cemented into the tap, extending about 1 in. above it. When the tap was in position the gassing tubes coincided with two holes in the top block which allowed access into each chamber. By rotating the tap the chambers could be simultaneously opened or closed, thus allowing fluids admittance to the chambers. The area of contact between the tap and the top block was also greased to ensure an air-tight joint (Pl. 2).

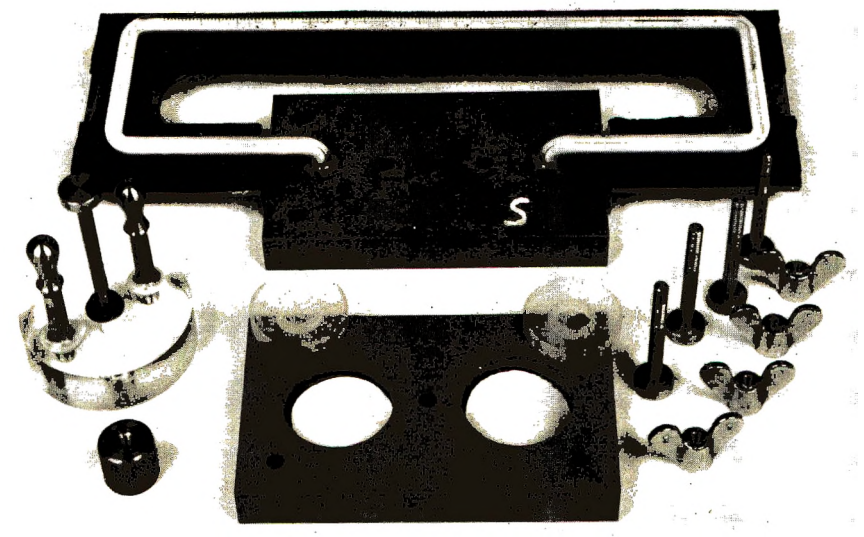
To measure skin respiration in micro-respirometers, thin slices of skin approximately 0·3 mm. thick were hand-cut from the bulges referred to under Skin Preparation and weighed on a torsion balance. For each respirometer 18–20 mg. was required. The skin was floated on the medium in one glass cup only of the respirometer, the other cup contained medium only. Potassium hydroxide was added to the filter-paper disk, indicator was placed in the capillary tube, and the two blocks were then screwed together with the tap open. The respirometer was then transferred to a water-bath at 37° C., the upper ends of the gassing tubes protruding above the water. When the apparatus had reached a uniform temperature, the tap was closed and recordings of oxygen uptake were made at 15–30 min. intervals.



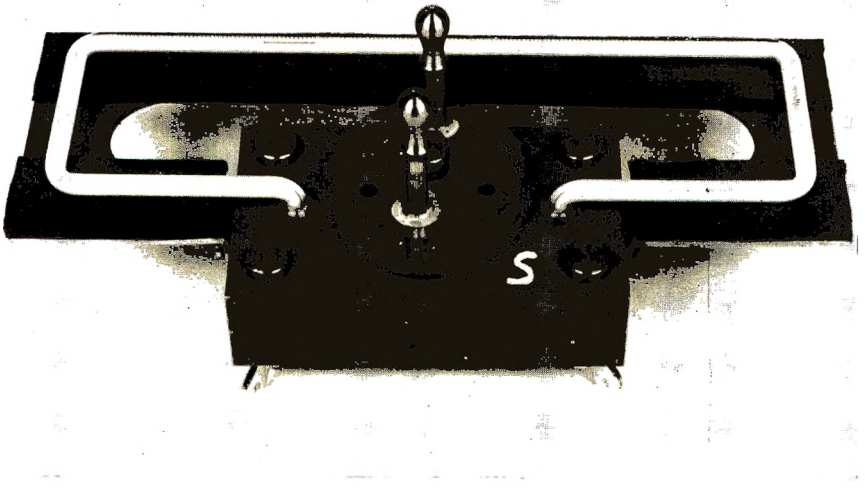
(*a*)



(*b*)



(*a*)



(*b*)

An experimental factor was calculated for each respirometer depending upon the weight of the skin sample and the calibration of the capillary tube. Oxygen uptake was expressed as μl. of oxygen per hour.

Respiration of pig skin was estimated in a medium containing antibiotic in a glass cup. When attempts were made to measure the respiration of the bacteria in pig skin, in the absence of antibiotic, bacteria left the skin to grow independently in the medium. To overcome this difficulty polythene cups were made with an inner compartment for the tissue, omitting the medium, and an outer compartment for 0·1 ml. water to provide a humid atmosphere. The polythene cups were 1·8 cm. diameter and 0·4 cm. deep, with an inner well of 0·8 cm. diameter.

Viable counts. The tissue was disintegrated by grinding it manually in a sterile Quickfit joint to liberate the bacteria. The debris was taken up in 5 ml. quarter-strength Ringer's solution containing 10% horse serum or penicillinase to inactivate germicide or penicillin respectively. Viable counts were then done in pour plates using nutrient agar containing 10% horse serum. Plates were incubated aerobically at 37° C. for 48 hr., after which colonies were counted.

Reagents. Streptomycin and sodium benzylpenicillin, as supplied by Glaxo Laboratories, Ltd., were used in aqueous solutions. Penicillinase, also supplied by Glaxo, was diluted as required in aqueous solution containing 5% serum to inactivate the phenol preservative. Krebs–Ringer phosphate was prepared as follows:

Solution A. Sodium chloride 0·9%, potassium chloride 0·046%, calcium chloride 0·040%, magnesium sulphate ($7H_2O$) 0·038%; in distilled water.

Solution B. M/10 disodium hydrogen phosphate, M/50 hydrochloric acid; in distilled water.

Both solutions were sterilized at 121° C. for 15 min. and mixed in the proportion 10·8 ml. (A) with 2·1 ml. (B). They were not mixed until required for use because the calcium tended to precipitate on mixing. Glucose was prepared at 5·0% in distilled water and sterilized at 121° C. for 15 min. Pig serum was obtained by collecting blood into a sterile container. After clotting, serum was withdrawn and centrifuged. Horse serum was obtained from Burroughs Welcome.

RESULTS

(*a*) *Differentiation between skin respiration and bacterial respiration*

It was important to know whether the skin itself was respiring, because if it was allowance for it would have to be made when bacterial respiration on the skin was measured.

To investigate this problem, respirometers with glass cups containing medium were first used. Pig skin was tested in medium of various compositions to discover (*a*) whether a substrate had to be provided for respiration to occur, and (*b*) whether streptomycin inhibited bacterial respiration thus allowing skin respiration to be detected. Streptomycin was chosen because it was included in the original medium (Cruickshank, 1954) to inhibit bacteria. The composition of the media is given in Table 1.

Respiration was measured between 0 and 3 hr. after which measurement was discontinued until the following morning. The respirometers were left in the water-bath overnight with the taps open. Recordings were then continued from 16 to 22 hr. Bacterial counts were done on the skin plus medium at the end of this period. In the absence of a suitable quenching agent for streptomycin reliance was placed on dilution to a level below bacteriostasis. The effects of variations in media can be seen in Table 1.

Table 1. *Effect of medium composition on respiration*

Medium	Pig serum (ml.)	Krebs-Ringer (ml.)	5% Glucose (ml.)	Strepto-mycin (500 units per ml.)	Bacterial count (per ml.)	Rate of O_2 uptake (μl./mg./hr.)
1	5	3	1	1	300,000	1·0
2	5	4	1	—	250,000,000	2·5
3	—	8	1	1	840,000	0·5
4	—	9	1	—	280,000,000	6·1
5	—	9	—	1	140,000	0
6	—	10	—	—	116,000,000	6·5

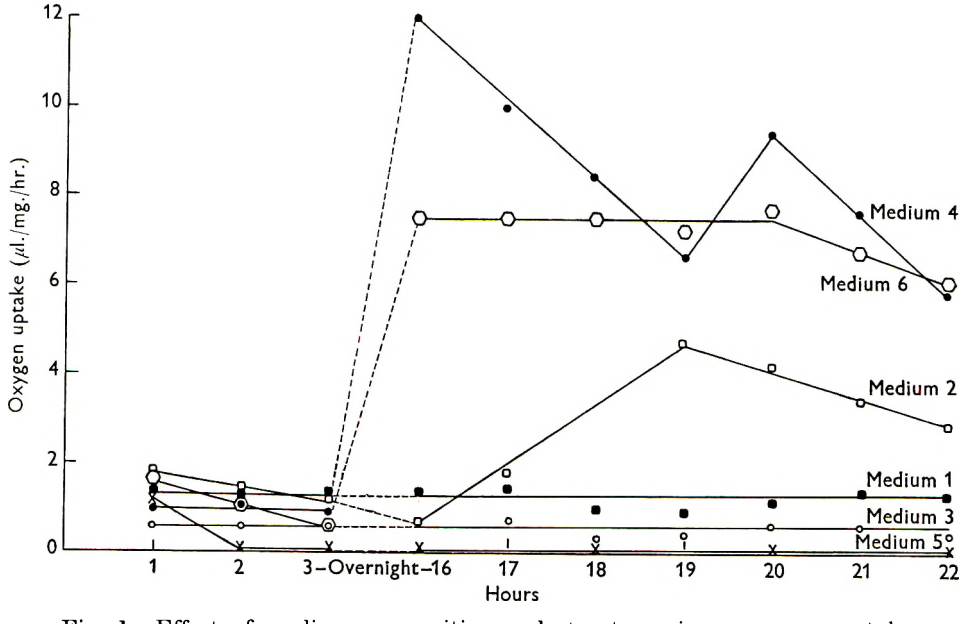


Fig. 1. Effect of medium composition and streptomycin on oxygen uptake by pig skin.

The table shows that in the presence of streptomycin the bacterial counts were low compared with those in its absence. This is reflected in the rate of oxygen uptake.

Correlation between bacterial counts and oxygen uptake could not be expected because the system of tissue in medium was variable and would be affected by many factors, including, for example, variable rates of growth between different kinds of bacteria. Therefore only gross differences could be shown. Fig. 1 shows

the respiration rates for the entire experiment. Media 1, 3 and 5 contained streptomycin, and the respiration was presumably therefore due to the skin itself and not to the bacteria growing on the skin. It is apparent that pig skin in Krebs–Ringer ceases respiring after 2 hr. (medium 5), although the addition of glucose and serum allowed respiration to continue for at least 22 hr.

From these results it was concluded that pig skin would not respire unless glucose and/or serum were present. To test this conclusion the following tests were done using the polythene cups, i.e. separating the skin from free fluid.

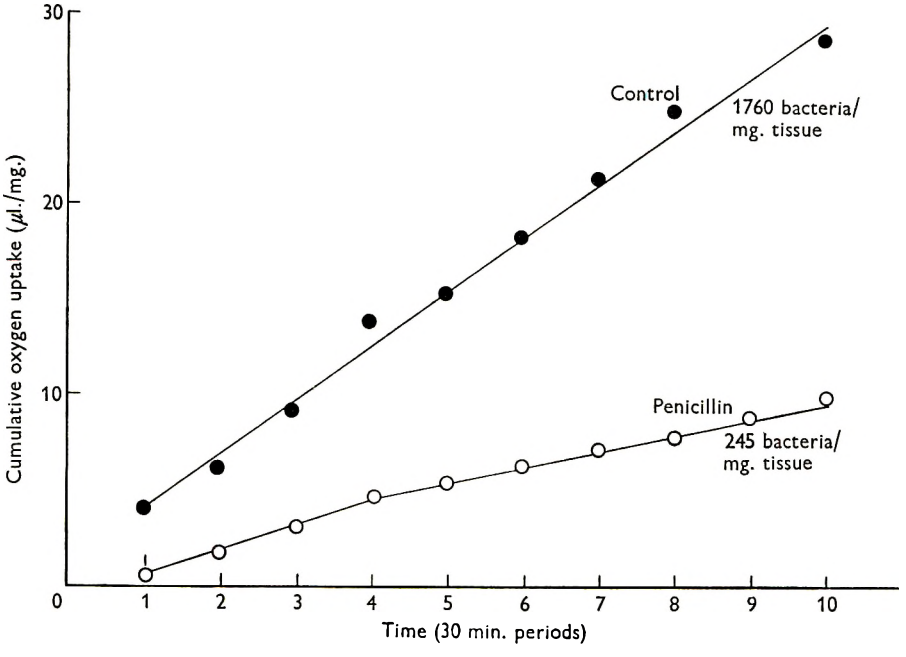


Fig. 2. Effect of penicillin on respiration of pig skin.

Pig skin was immersed 5 times in 10% soap solution at 42° C. for cleaning purposes, after which either water or penicillin at 150,000 units/ml. was dropped on its surface and allowed to evaporate at room-temperature. Penicillin was used instead of streptomycin in these experiments, because it is non-toxic to skin (Lawrence, 1959*b*), but toxic to the skin flora; it has the great advantage that it can be inactivated by penicillinase after the respiration rate has been measured, so that surviving bacteria can be counted without carry-over problems. Samples of the skin were placed in the inner well of the polythene cups without medium and their respiration measured over 5 hr. periods. They were then disintegrated for bacterial counts.

An example of the results obtained is shown in Fig. 2 in which cumulative oxygen uptake is plotted against time. In the absence of penicillin the respiration rate was much higher than in its presence and this difference was reflected in the viable counts, also shown in Fig. 2. It appears that most of the respiration was due to bacteria.

The time for these experiments was then extended to determine whether penicillin-treated skin showed respiratory activity on further incubation. Fig. 3 shows that when measurements of respirations were started after 20 hr. incubation penicillin-treated skin was not respiring, and this was substantiated by bacterial counts done on the skin samples.

(*b*) *Relationship between oxygen uptake and bacterial numbers*

If the effect of germicides on respiration is to be measured it is important to know whether the level of oxygen uptake truly reflects the number of viable bacteria present, as suggested by the data in Figs. 1–3. Skin samples from 5 hr. respiration experiments were therefore disintegrated and the viable bacteria counted. The figures for oxygen uptake were obtained by combining the last three readings obtained from each skin sample. Good correlation was obtained in individual experiments, as shown by the results of a typical experiment given in Fig. 4. The results of five such experiments, giving a total of 95 observations each of oxygen uptake and viable counts, were examined statistically.

It was found that in general there was some relationship and this could be represented by straight lines for each test. However, the slope varied from day to day and the lines were at different levels for each test, and therefore a general relationship was not obtained when data from all five experiments was combined.

(*c*) *Variations between micro-respirometers and between pig skin samples*

Experiments designed to determine the variation between respirometers and between skin samples taken from different trotters included nine trotters examined on each of 5 days, two samples being taken from each trotter. Oxygen uptake was recorded at 30 min. intervals for 5 hr.

Differences between respirometers were not significant and although differences may have been present the results indicated that they were not large. In subsequent experiments the respirometers were therefore statistically randomized so that any slight differences could be eliminated.

Comparison of the respiration rate of skin samples from different trotters showed highly significant differences. To compare treatments it was therefore necessary to use two skin samples from the same trotter thus increasing accuracy.

To examine variation between samples from the same trotter each one was divided into six areas in a straight line along the back of the pastern joint. Measurement of respiration rates showed that the samples did not differ significantly. From these results it was concluded that skin samples could be taken from any of the sites within a trotter and a similar rate of respiration could be expected.

(*d*) *Respiration during 24 hr. period*

Most of the preliminary work was done during 5 hr. periods, but these experiments (Fig. 3) indicated that the use of longer periods would be preferable. To examine this problem nine pigs' trotters were used on each of 5 days. Before taking skin samples for respirometry the skin was immersed in 10 % soap solution,

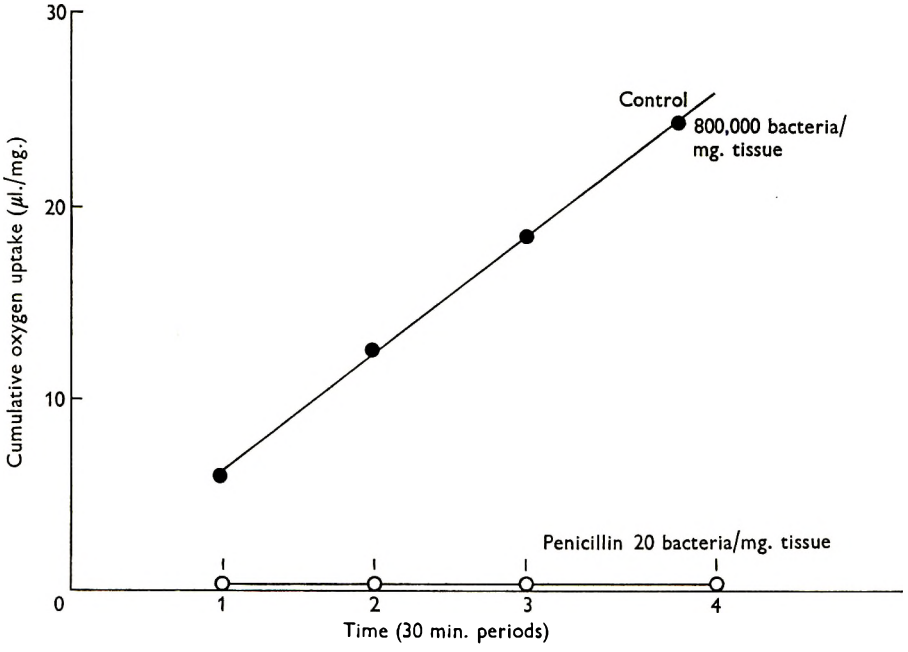


Fig. 3. Effect of penicillin on respiration of pig skin after 20 hr. incubation.

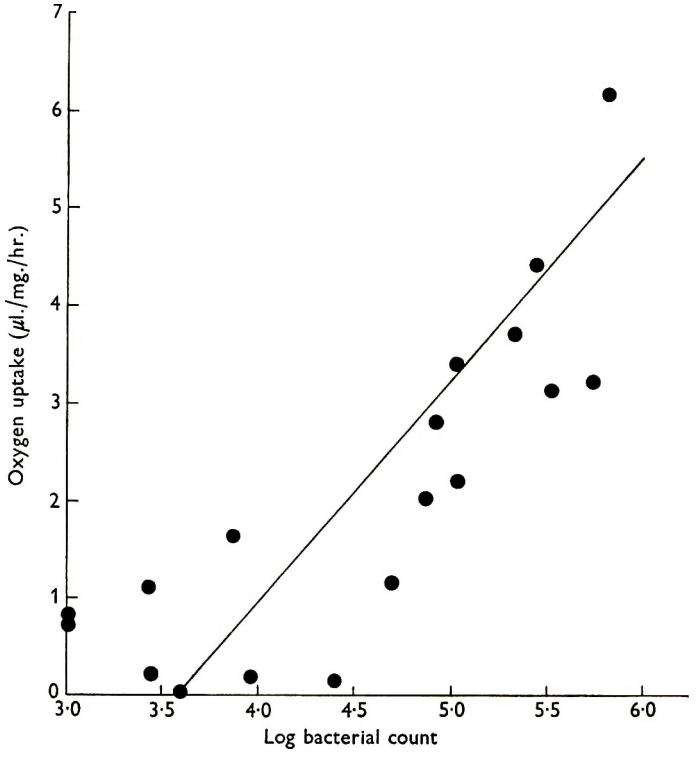


Fig. 4. Correlation between oxygen uptake of pig skin and number of bacteria present.

whilst held on the wooden blocks to ensure cleanliness. Respiration was recorded at 30 min. intervals for 24 hr. Fig. 5 shows the rate of oxygen uptake in μl. per hour plotted against time. In the example shown in Fig. 5 there was an increase in respiration rate for about 9 hr. after which the increase was much slower. Another, similar, experiment indicated an increase up to 6 hr. also followed

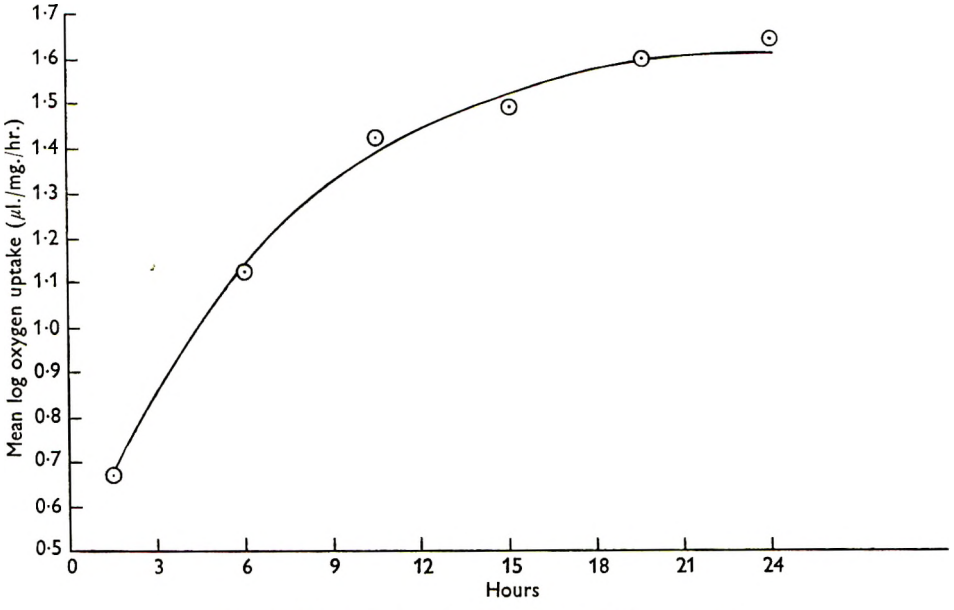


Fig. 5. Pig-skin respiration during 24 hr. period.

by a slower increase. The period during which the respiration did not increase rapidly, say from 9 hr. onwards, seems a suitable time for testing germicides because respiration rates are becoming maximal and therefore differences in rates will be easier to detect.

The application of these findings to a technique for testing germicides on pig skin will be given in a subsequent paper.

DISCUSSION

The number of bacteria on skin is usually determined by washing to remove them and doing viable counts on the wash water. The use of micro-respirometers enables their respiration to be measured *in situ*. However, it is important that the bacterial respiration in skin samples be differentiated from skin respiration, otherwise it would be impossible to decide whether added reagents were causing a reduction in bacterial or skin respiration or both.

In this investigation it has not been possible conclusively to demonstrate presence or absence of skin respiration because undamaged, sterile skin was not available. Experiments in which pig-skin samples were suspended in a medium containing streptomycin showed that there was respiration in the presence of glucose and serum. In the absence of these substances respiration (in Krebs–

Ringer phosphate plus streptomycin) continued for only about 2 hr. This respiration was of a lower order than that obtained on addition of glucose and serum which allowed continued activity for at least 22 hr. It is therefore probable that the initial activity in Krebs–Ringer phosphate is residual and the skin requires added substrate for continued respiration.

Penicillin-treated skin in polythene cups also respired slightly at the start of the experiments but this had stopped by the following morning. This is not likely to have been due to penicillin inhibiting skin respiration (Lawrence, 1959*b*), and it can therefore be concluded that the level of skin respiration, if present, is negligible and does not require differentiation from bacterial respiration when the 9–24 hr. period is used.

Bacteria on human skin have been differentiated into 'transient' and 'resident' (Price, 1938). Transients are loosely attached to the skin and are easily removed, whereas residents compose a constant population not so easily disposable. This division could also be applied to pig skin and it is the resident bacteria which remain in the skin samples used for respirometry, transients being removed by immersing the skin in soap solution before cutting slices.

Uneven distribution of the resident bacteria on the pig skin would obviously cause the variation of respiration rates between skin samples. It can be assumed that they are probably evenly distributed along the surface of the trotter otherwise there would be variation between samples from the same trotter, which is not the case. However, there is considerable variation in the respiration rates of skin samples taken from different trotters. This situation is similar to that obtained on human hands where there are large differences between subjects in the number of bacteria obtained after washing and rinsing (Hurst *et al.* 1960). The accuracy of results from hand-washing tests can be increased by increasing the number of subjects taking part. A similar situation exists when respiration rates of pig skin samples are measured.

Variation between trotters may also be due to differences in the thickness of skin samples. The variable contours of the skin (e.g. ridges etc.) make it impossible to get exactly comparable samples from different trotters. This is particularly true of hand-cut sections. These differences in thickness can result in two samples of identical weight having very different surface areas. Even if the surfaces were equally contaminated with bacteria per unit area, wide differences in numbers of bacteria would be obtained. Also, the available oxygen supply will depend on skin thickness.

Another difference observed between trotters is the degree of hydration. This will probably have a profound effect on the skin bacteria (Blank & Dawes, 1958).

Correlation between oxygen uptake and bacterial counts was obtained for individual days after 5 hr. incubation. Whether this correlation would hold after longer periods of incubation is not known. During prolonged incubation the respiration rate increased rapidly for 6–9 hr. followed by a slower increase up to 24 hr. After 6–9 hr. multiplication may continue while respiration is limited by the supply of fermentable substrate; another explanation would be that the bacteria have reached a state of equilibrium.

Even though the mechanism of this system is not fully understood it is being used to estimate the effect of skin germicides on bacterial respiration.

SUMMARY

1. The respiration of bacteria normally present in thin slices of skin taken from pig's trotters has been measured in Cruickshank micro-respirometers.

2. These respirometers were originally designed to measure skin respiration in a medium including antibiotic to inhibit bacterial activity. The skin and medium was contained in a glass well. Using pig skin in a similar system without antibiotic, bacteria left the skin to grow independently in the medium. To overcome this difficulty polythene cups were made with an inner compartment for the tissue without medium and an outer compartment for water which provided a humid atmosphere.

3. Using polythene cups without medium the skin respiration was neglible, bacterial respiration was measured *in situ* and correlation between oxygen uptake and bacterial counts was obtained. This correlation was valid within experiments only.

4. Respiration of skin samples taken from adjacent areas of pig's trotter was similar, but there was large variation between samples from different trotters.

5. Respiration due to bacteria increased rapidly for 6–9 hr., followed by a slower increase. This may be due to a limited food supply or to a state of equilibrium.

My thanks are due to Dr A. Hurst and Mr B. M. Gibbs for helpful discussions and to Mr A. Marlow for valuable technical assistance.

REFERENCES

BAIRD-PARKER, A. C. (1962). The occurrence and enumeration, according to a new classification, of micrococci and staphylococci in bacon and on human and pig skin. *J. appl. Bact.* **25**, 352.

BLANK, I. H. & DAWES, R. K. (1958). The water content of the stratum corneum. IV. The importance of water in promoting bacterial multiplication on cornified epithelium. *J. invest. Derm.* **31**, 141.

CRUICKSHANK, C. N. D. (1954). Continuous observation of the respiration of skin in vitro. *Exp. Cell Res.* **7**, 374.

CRUICKSHANK, C. N. D., TROTTER, M. D. & COOPER, J. R. (1957). Studies on the carbohydrate metabolism of skin. *Biochem. J.* **66**, 285.

HURST, A., STUTTARD, L. W. & WOODROFFE, R. C. S. (1960). Disinfectants for use in bar-soaps. *J. Hyg., Camb.*, **58**, 159.

LAWRENCE, J. C. & RICKETTS, C. R. (1957). The metabolic uptake of sulphate ions by skin. *Exp. Cell Res.* **12**, 633.

LAWRENCE, J. C. (1959*a*). Some effects of staphylococcal and streptococcal toxins upon mammalian skin in tissue culture. *Brit. J. exp. Path.* **40**, 8.

LAWRENCE, J. C. (1959*b*). The comparative toxicity of antibiotics to skin. *Brit. J. Pharmacol.* **14**, 168.

PRICE, P. B. (1938). The bacteriology of normal skin; a new quantitative test applied to the study of the bacterial flora and the disinfectant action of mechanical cleansing. *J. infect. Dis.* **63**, 301.

J. Hyg., Camb. (1963), **61**, 283
Printed in Great Britain

A respirometer technique for estimating germicidal activity on skin

By R. C. S. WOODROFFE
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire

(*Received* 21 *February* 1963)

INTRODUCTION

Germicidal soaps are used to reduce the number of viable bacteria on skin. It is advisable that the number be minimal for two reasons. First, various workers have shown that *Staphylococcus aureus* is carried on skin (Miles, Williams & Clayton-Cooper, 1944; Williams, 1946; Hare & Thomas, 1956; Hare & Ridley, 1958). With the chance that some of them may become invasive a low bacterial population is preferred. Secondly, a cosmetic reason is that body-odour can be caused by bacterial decomposition of cutaneous secretions (Hurley & Shelley, 1960).

Methods for estimating the *in vivo* effect of these soaps on the bacterial flora of skin have been reviewed and described elsewhere (Hurst, Stuttard & Woodroffe, 1960). These methods require panels of human volunteers who use germicidal soap or germicide-free soap for a week, after which the numbers of bacteria washed off their hands are estimated in the laboratory. Bacterial counts are done on the wash water and in this way percentage reductions in skin flora caused by the germicidal soap can be estimated. Such investigations are tedious and therefore a realistic *in vitro* screening test for germicidal activity on skin is required.

Germicides contained in soap are applied to the skin during washing. They are retained on skin and remain on it after the soap has been rinsed off and are likely to accumulate and have a continuous activity thus reducing bacterial numbers during the course of time. Because retention on skin is probably the most important property of active skin germicides it has been used in various screening tests as an indication of potential antibacterial activity.

Several *in vitro* tests designed to demonstrate retention of germicides on skin have been described. Some investigators have applied the germicide to skin and then recovered it with solvents for assay (Fahlberg, Swan & Seastone, 1948, Compeau, 1960). Others have applied germicide to gelatin film or skin and detected its presence by demonstrating zones of inhibition on seeded agar surrounding the test material (Bechtold, Lawrence & Owen, 1955; Vinson, 1961). Such tests are useful in demonstrating the presence of germicide on skin even though its antibacterial activity whilst on the skin itself is not demonstrated. However, hand-washing tests also have their limitations. For instance, one weakness is the assumption that the antibacterial effect of germicide whilst on skin is reflected by a decrease in the number of viable bacteria coming off during a standardized wash. This assumption is difficult to prove or disprove without bacteriological examination of skin samples taken from hands. An *in vitro* test which could measure

germicidal activity actually on skin (*in situ*) would therefore be valuable and these considerations led to the present work.

The respiration of bacteria normally found in pig skin has been measured (Woodroffe, 1963), using a modification of micro-respirometers originally designed to measure skin respiration (Cruickshank, 1954). In this system skin respiration is negligible or absent and therefore only bacterial respiration is measured. In the present investigation this technique has been used to estimate the antibacterial activity of germicides applied to pig skin.

METHODS AND MATERIALS

Preparation of skin

Pig skin was obtained from pigs' trotters and prepared for soap treatment as previously described (Woodroffe, 1963).

Germicides

Ten per cent solutions of Lux toilet soap were prepared in beakers and used at 42° C. Alcoholic solution of germicide was added to these as required. Germicides used in this investigation were: 2,2'dihydroxy, 3,5,6,3',5',6'-hexachlorodiphenylmethane (G11), 3,4,4'-trichlorocarbanilide (T_3CC), 3,5,4'-tribromosalicylanilide (T_3BS), 2,2-thiobis (4,6-dichlorophenol) (Actamer), Irgasan CF3,* 3,5,3',4'-tetrachlorosalicylanilide (T_4CS).

While these investigations were in progress, it became known that T_4CS was a photo-sensitizing agent; its use on skin is therefore clearly undesirable (Wilkinson, 1961). However, the results obtained by using this germicide are included because this investigation was concerned only with the antibacterial activity of germicides and not their toxicity.

Exposure to germicides

To estimate the effect of a germicidal soap on pig skin each experiment was done on 5 consecutive days and nine trotters were used each day. Both boards of skin, test and control, were immersed for 30 sec. in soap solution up to 20 times, rinsing between each immersion for 15 sec. After 1, 4, 8, 12, 16 and 20 immersions samples of skin approximately 0·3 mm. thick were cut from the bulges, weighed on a torsion balance and placed in respirometers. Each respirometer received a total of approximately 20 mg. from one bulge only.

Positions on the boards were randomized statistically, as also were the respirometers, to allow for variation between tissue samples and between respirometers. These randomizations covered the entire 5 days. Respiration was recorded at 30 min. intervals at 37° C.

Estimation of retained germicide

The concentration of germicide retained on pig skin was estimated by immersing the skin in germicide soap solution followed by ether extraction and microbiological assay (Fahlberg *et al.* 1948).

* J. R. Geigy, Basle.

To do this, all the skin from a trotter was stretched over the open end of a 25 ml. spoutless beaker and secured with crocodile clips attached to rubber bands. Thus a drum, 7·5 cm. in diameter, was obtained which could be immersed in soap solution.

To apply the germicide, pig skin was immersed in 50 ml. germicidal soap solution for 60 sec. followed by rinsing under warm running water for 15 sec. This was done for 1 to 20 immersions depending upon the number required.

Germicide was reclaimed from the skin by immersion in 30 ml. ether for 2 min. The concentration removed was estimated by evaporating the ether and then doing a microbiological assay on the residues.

RESULTS

(i) *Effect of germicide soap treatments during a 5 hr. test period*

The effect on respiration of 4, 8, 12, 16 and 20 immersions in soap solution containing 400 μg./ml. T_4CS was determined, and compared with 20 immersions in germicide-free soap. The results showed that during the 5 hr. period during which respiration was recorded there was a significant rise in the rate of oxygen uptake from the first to the last readings though this did not vary between numbers of immersions. There was also a suggestion that this rise may have been greater for the control skin samples than for the tests. The mean uptake during all ten of the 30 min. recordings were used in the analysis and the results obtained are given in Table 1.

Table 1. *Mean (log) rate of oxygen uptake (μl./mg./hr.) after immersions in T_4CS soap*

	Immersions					All immersions
	4	8	12	16	20	
Test	0·515	0·471	0·525	0·485	0·406	0·481
Control	—	—	—	—	0·656	0·656

The mean of all the skins treated with germicide was significantly less than that for the controls. There was a significant reduction in respiration rate after 4 immersions but further immersions did not add to it.

The conclusion reached after examining these results was that there was sufficient germicide on the skin after 4 immersions to reduce the rate of respiration and that because further immersions did not change the reduction the amount of germicide absorbed after 8 or more immersions was probably no greater than after 4 immersions. To challenge this conclusion the concentration of T_4CS absorbed on pig skin was estimated after various numbers of immersions.

(ii) *Accumulation of T_4CS on pig skin*

The concentration of germicide recovered from pig skin was estimated after 1, 4, 8 and 20 immersions in 10% soap solution containing 400 μg./ml. T_4CS.

Sixteen skins were used for each set of immersions and the results averaged. The amount of germicide extracted with ether can be seen in Table 2.

Table 2. *Accumulation of T_4CS on pig skin*

No. of immersions	T_4CS extracted (μg.)
1	2·73
4	3·75
8	8·40
20	13·50

These results show that the amount of germicide retained on skin increases with the number of immersions.

The fact that T_4CS accumulated on skin indicated that the results obtained from respirometry were misleading. The observation that oxygen uptake at the end of a 5 hr. period probably increased in control but not in germicide-treated skin indicated that the effect of germicide treatment should be measured over a longer period of time. Experiments were therefore designed to test this hypothesis.

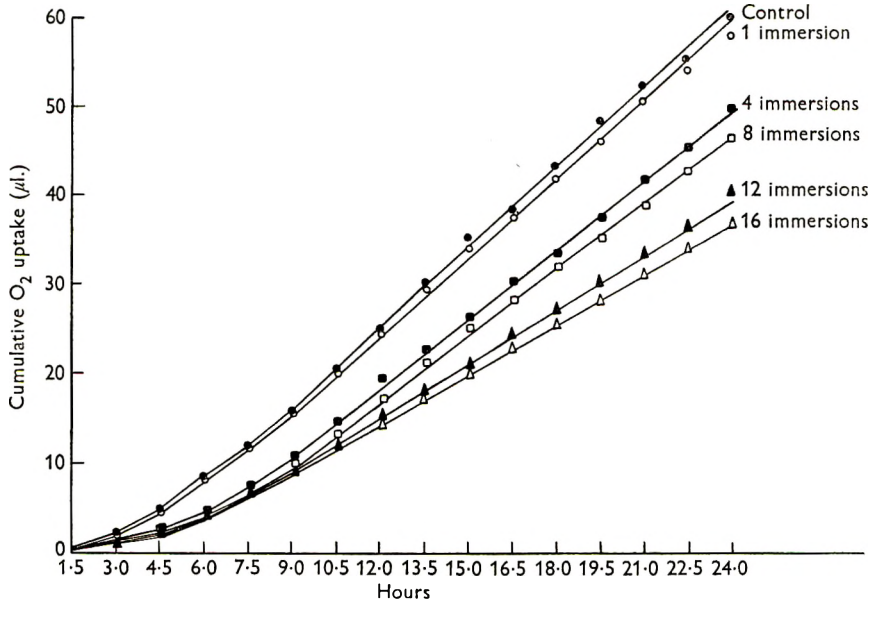


Fig. 1. Oxygen uptake of pig skin after treatment with soap solution containing 400 μg./ml. T_4CS

(iii) *Effect of germicide soap treatments during a 24 hr. test period*

The effect on respiration of 1, 4, 8, 12 and 16 immersions in soap solution containing 400 μg./ml. T_4CS was determined by recording respiration rates at 30 min. intervals during 24 hr. periods.

Respiration can be seen in Fig. 1 where cumulative oxygen uptake of tests and controls are plotted against time. The lines are curved up to about 9 hr. and there-

after become straight, i.e. between 9 and 24 hr. the rate of oxygen uptake does not increase either for the control or for each number of immersions. The differences between control and tests are greater than during the first 9 hr.

The first part of the statistical analysis of the results compared the mean respiration rate of control skins with the mean of the germicide-treated skins, irrespective of the number of immersions. To simplify the procedure the analysis was performed for each of six periods each of $1\frac{1}{2}$ hr., spaced at 3-hourly intervals, on the logarithm of the rate of oxygen uptake. Table 3 gives the mean log rates.

Table 3. *Mean (log) oxygen uptake of skin samples*

Period (hr.)	0–$1\frac{1}{2}$	$4\frac{1}{2}$–6	9–$10\frac{1}{2}$	$13\frac{1}{2}$–15	18–$19\frac{1}{2}$	$22\frac{1}{2}$–24
Control skin	0·663	1·127	1·429	1·484	1·603	1·627
Germicide-treated skins	0·662	0·861	1·121	1·235	1·312	1·368

For the first period (0–$1\frac{1}{2}$ hr.) the difference between control and test was not significant, but for later periods the germicide-treated skins showed a lower rate of uptake than the control. Further analysis showed that the treatment effects were the same for each of the periods analysed (between $4\frac{1}{2}$ and 24 hr.). Because of this, all the rates of oxygen uptake from $4\frac{1}{2}$ to 24 hr. were included in the calculation of results. Percentage reductions in oxygen uptake, shown in Table 4, were then calculated for each set of immersions.

Table 4. *Effect of number of immersions in T_4CS soap*

No. of immersions	Percentage reductions	Confidence limits
14	36·79*	58·37: 4·02
	33·18	56·00:–1·46
8	32·47	55·53:–2·54
12	47·64*	65·52:20·49
16	59·69*	73·45:38·79

* Significant reduction ($P = 0{\cdot}05$).

Reduction required for significance was 34·2%. The values for different numbers of immersions did not differ significantly.

The slopes of the lines in Fig. 1 are subject to experimental errors because of the variations between individual pig skins. Consequently, the slopes for different numbers of immersions did not differ significantly, although the graph indicates that the true slope may decrease as the number of immersions increases.

These calculations indicated that germicidal activity could be estimated at any time between $4\frac{1}{2}$ and 24 hr. after the experiment commenced. Later experiments were therefore done using $16\frac{1}{2}$–21 hr. as the test period. Other germicides in soap were then examined.

(iv) *Effect of various germicidal soaps on respiration*

The effect of six different germicides on skin was estimated after 0–20 immersions in soap solutions, germicides were used at 400, 1000 and 2000 μg./ml. These investigations were done to discover whether the anti-bacterial effect of other skin

germicides was measurable in this system. No attempt was made to compare activities. Results are given in Table 5.

Table 5. *Percentage reductions in oxygen uptake caused by various germicides*

Germicide	Concentration (μg./ml.)	No. of immersions 1	4	8	12	16	20
T_3BS	400	−3	0	20	17	8	18
	1000	−10	12	79*	49*	48*	23
	2000	−29	24	46*	64*	38*	86*
G 11	400	1	−20	−22	17	12	15
	1000	−2	0	35	−38	24	19
	2000	−68	−36	−21	35	−4	36
Actamer	400	10	−15	−34	8	32	−6
	1000	−26	−22	−28	24	−1	36
	2000	6	15	22	17	17	14
Irgasan CF 3	400	19	16	−5	11	27	33
	1000	−1	20	12	35	67*	54*
	2000	4	15	32	46*	48*	−9
T_3CC	400	−44	14	−15	20	28	−14
	1000	−6	−43	5	−4	−51	−15
	2000	−58	−12	48*	22	24	12
T_3CS	400	36*	33	32	47*	59*	—
	1000	−2	32	57*	63*	80*	75*
	2000	−27	45*	50*	83*	84*	93*

* Significant reduction ($P = 0{\cdot}05$).

Reduction required for significance at 400μg./ml., 34·2%; at 1000 μg./ml., 37·0%; at 2000μg./ml., 41·4%.

Among the germicides which showed no activity were G 11, Actamer and T_3CC, whereas the rest all reduced the respiration rate even though this was not very consistent. Generally speaking an increase in germicidal concentration resulted in increased antibacterial activity.

DISCUSSION

The respirometry results presented here show that the activity of germicides on skin bacteria can be measured using excised skin. The technique can therefore be used to screen new skin germicides for their ability to attack bacteria whilst on skin, also to determine the numbers of immersions required to cause a significant reduction in bacterial respiration. The importance of affecting respiration has, however, yet to be assessed in relation to other properties of skin germicides.

We have shown that T_4CS accumulates on skin, also that reduction in respiration increases with numbers of immersions and germicide concentration. If the results on known germicides can be related to panel tests under user conditions, i.e. hand-washing test results, useful predictions about the possible results with new substances might be made. However, this is not yet possible because the results obtained from respirometry are not readily understood when considered in relation

to hand-washing test results. G 11, Actamer and T_3CC appear to have little or no activity in respirometers, whereas in hand-washing tests they cause significant reductions in bacterial flora (Hurst, *et al.* 1960). What relationship then, if any, do the results from respirometers have to the effect of germicidal soaps on the bacterial flora of human skin?

The ability of germicidal soaps to reduce the bacterial flora of hands is usually assessed at the end of 1 week's use; these are expressed as percentage reductions and are the only figures available for comparison with respirometer results. All the germicides tested in respirometers have previously been examined in hand-washing tests when they were shown to be active against the bacterial flora of skin, yet in respirometers T_3CC, Actamer and G 11 were inactive, whereas T_3BS, Irgasan CF 3, and T_4CS showed good activity. This difference could be due to the former group having a different mode of action to the latter. Information about the mode of action of most of the germicides tested is, however, very scant, and only in the case of G 11 is there reported work (Gould, Bosniak, Niedleman & Gatt, 1953; Gould, Frigerio & Lebowitz, 1955; Adams, 1958; Adams & Hobbs, 1958; Joswick, 1961). It is difficult therefore to assess this possibility. The absence of activity might also be explained by a difference in the speed of action of these germicides. Results from hand-washing tests do not show when, during the course of a 7-day test period, the germicide caused a significant reduction. Two different germicides might, therefore, appear of equal activity after 7 days yet one of them may have acted more rapidly than the other. Daily assessment of germicidal soap activity during hand-washing tests would therefore be of value when attempting to correlate results from respirometers with those from hand-washing tests. There is some indication (unpublished data) that the second explanation is the most likely.

Results of screening tests must be interpreted with caution. The respirometer technique has the drawback that bacteria incapable of growth may continue to respire and it is not yet known at what rate a skin germicide must kill in order to have a satisfactory effect on skin flora *in vivo*.

SUMMARY

1. A technique for measuring the antibacterial activity of germicides on pig skin in micro-respirometers has been described.

2. Using this technique the activity of the following germicides has been estimated: 2,2-dihydroxy, 3,5,6,3′,5′,6′-hexachlorodiphenylmethane, 3,4,4′-trichlorocarbanilide, 3,5,4′tribromosalicylanilide, 2,2′-thiobis (4,6-dichlorophenol), Irgasan CF 3*, 3,5,3′,4′-tetrachlorosalicylanilide.

3. The results obtained have been discussed in relation to hand-washing test results (Hurst *et al.* 1960).

My thanks are due to Dr A. Hurst and Mr B. M. Gibbs for helpful discussions and to Mr A. Marlow for technical assistance.

* J. R. Geigy, Basle.

REFERENCES

ADAMS, J. B. (1958). The mode of action of chlorinated bisphenol antibacterials. Part I. Metal chelates of hexachlorophene and thiobisdichlorophenol. *J. Pharm., Lond.*, **10**, 507.

ADAMS, J. B. & HOBBS, M. (1958). The mode of action of chlorinated bisphenol antibacterials. Part II. Biological studies. *J. Pharm., Lond.*, **10**, 516.

BECHTOLD, C. L., LAWRENCE, E. A. & OWEN, E. M. (1955). *In vitro* test for predicting the effectiveness of antibacterial agents in soap. *Proc. Sci. Sect. Toilet Goods Ass.* no. 24, 21.

COMPEAU, G. M. (1960). The adsorption of dodecylbenzenesulfonate and hexachlorophene on skin. *J. Amer. pharm. Ass. (Sci. ed.)*, **49**, 574.

CRUICKSHANK, C. N. D. (1954). Continuous observation of the respiration of skin *in vitro*. *Exp. Cell. Res.* **7**, 374.

FAHLBERG, W. J., SWAN, J. C. & SEASTONE, C. V. (1948). Studies on the retention of hexachlorophene (G 11) in human skin. *J. Bact.* **56**, 323.

GOULD, B. S., BOSNIAK, M. A., NIEDLEMAN, S. & GATT, S. (1953). Effect of hexachlorophene and related bisphenolic compounds on the dehydrogenases and cytochrome system of *Bacillus subtilis* and *Escherichia coli*. *Arch. Biochim. Biophys.* **44**, 284.

GOULD, B. S., FRIGERIO, N. A. & LEBOWITZ, W. B. (1955). The action of bisphenolic compounds on succinoxidase, cytochrome *c* oxidase, and lactic dehydrogenase of animal tissue. *Arch. Biochim. Biophys.* **56**, 476.

HARE, R. & RIDLEY, M. (1958). Further studies on the transmission of *Staphylococcus aureus*. *Brit. med. J.* i, 69.

HARE, R. & THOMAS, C. G. A. (1956). The transmission of *Staphylococcus aureus*. *Brit. med. J.* ii, 240.

HURLEY, H. J. & SHELLEY, W. B. (1960). *The Human Apocrine Sweat Gland in Health and Disease*, p. 81. Oxford: Blackwell Scientific Publications.

HURST, A., STUTTARD, L. W. & WOODROFFE, R. C. S. (1960). Disinfectants for use in bar-soaps. *J. Hyg., Camb.*, **58**, 159.

JOSWICK, H. L. (1961). Mode of action of hexachlorophene. Ph.D. Dissertation, University of Michigan.

MILES, A. A., WILLIAMS, R. E. O. & CLAYTON-COOPER, R. (1944). The carriage of *Staphylococcus (pyogenes) aureus* in man and its relation to wound infection. *J. Path. Bact.* **56**, 513.

VINSON, L. (1961). *In vitro* tests for measuring antibacterial activity of toilet soaps and detergent bars. *J. Pharm. Sci.* **50**, 827.

WILKINSON, D. S. (1961). Photodermatitis due to tetrachlorosalicylanilide. *Brit. J. Derm.* **73**, 213.

WILLIAMS, R. E. O. (1946). Skin and nose carriage of bacteriophage types of *Staphylococcus aureus*. *J. Path. Bact.* **58**, 259.

WOODROFFE, R. (1963). The measurement of bacterial respiration on pig skin with micro-respirometers. *J. Hyg., Camb.*, **61**, 273.

J. Hyg., Camb. (1963), **61**, 291
Printed in Great Britain

A seventeen-year survey of the ringworm flora of Birmingham

By GWENDOLINE I. M. CARLIER
The Skin Hospital, Birmingham

(*Received* 26 *February* 1963)

INTRODUCTION

The main object of this survey has been to ascertain the sources of infection with a view to their eradication. What measure of success has been attained will appear from the facts and figures here set down. A detailed record of every case referred to the laboratory has been kept since 1 January 1945. While it would be idle to suggest that every case of ringworm in the city has been seen at this hospital (because an unascertainable number of cases must undoubtedly have been treated elsewhere), it is reasonable to claim that the body of investigations here discussed constitutes a reliable indication of the distribution of species, their relative frequency and their topographical concentration.

AREA

For the delivery of mail the 80 square miles that comprise the city of Birmingham are divided into thirty-four postal districts. Because patients usually give their addresses in this form the postal district, rather than the ward, was adopted as the most convenient unit for the investigation of the local ringworm flora at the outset of the work. Though no population statistics for the postal districts could be obtained, this proved nevertheless to have been a fortunate choice, for the ward boundaries suffered drastic revision in 1949, whereas the postal districts have remained unchanged since their inception. Comparison of maps 1 and 2 yields a good general idea of the population pattern of the city.

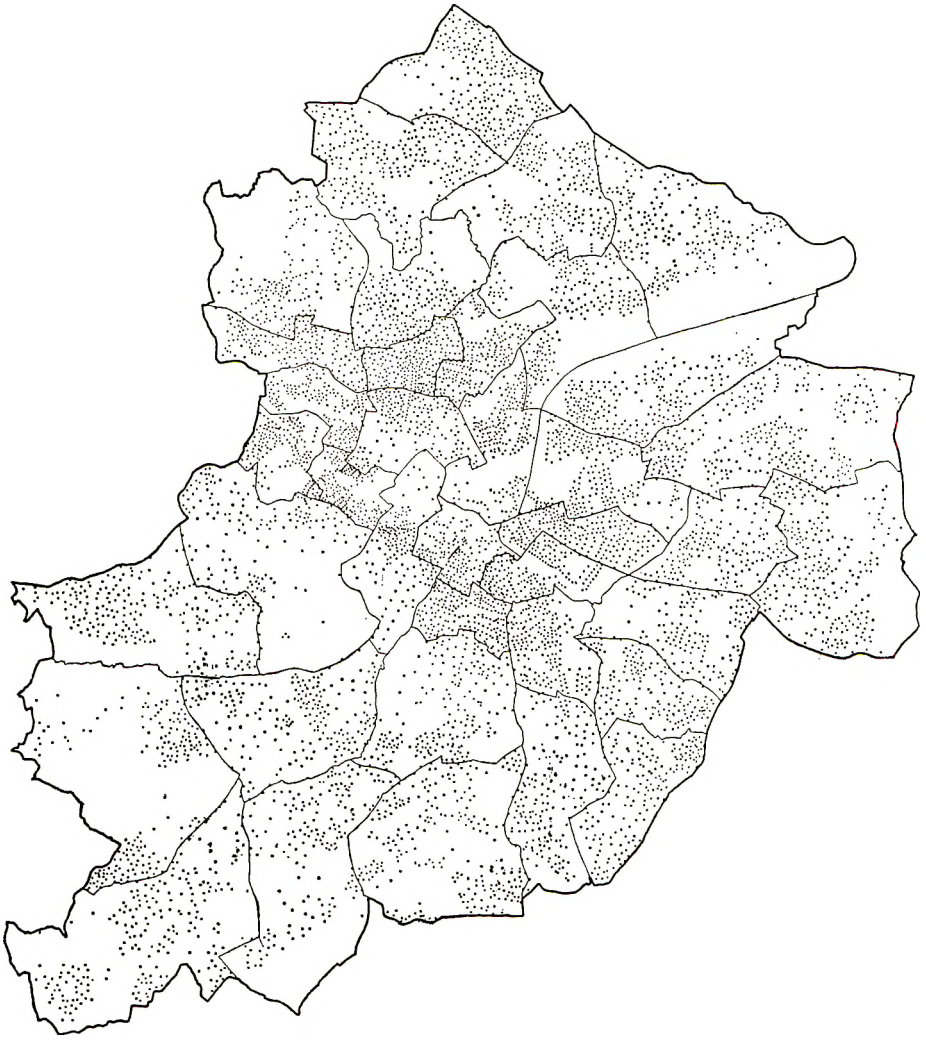
NOMENCLATURE

In the preliminary survey (Carlier, 1954) the recommendations of the Medical Mycology Committee of the Medical Research Council (1949) were followed. In a later edition (1958) a few modifications were introduced. These have been substituted where necessary in the present work.

TECHNIQUE

Infected scalps are inspected under filtered ultra-violet light in the consulting-room and the result communicated to the laboratory. Here the suspected patches are swabbed with petroleum ether which, as it evaporates, produces an appearance of hoar-frost on every infected hair. This device is effective in exposing 'endothrix' infections, as well as the fungi that fluoresce under ultra-violet light, is painless to the patient and saves time, inasmuch as viable specimens can be extracted with the greatest ease in a few minutes.

Samples of the glabrous skin and the nails are obtained by scraping. All specimens, hairs, nails or scales, are divided into two portions, one for immediate examination in warm 20 % KOH under the microscope and the other 'planted' on proof medium and incubated at 20–24° C. for as long as is needed for identification or for at least a fortnight before being discarded as negative. In the case of nail-



Map 1. Map of Birmingham showing density of the total population, 1951–52. One dot represents 150 persons. Reproduced by courtesy of the City Statistician.

scrapings, it has been our practice in recent years to make the primary culture on proof medium containing 0·5 g. of cycloheximide per litre to inhibit the growth of any green-mould spores which the specimen may be harbouring (Georg, Ajello & Papageorge, 1954).

In the majority of cases the cultures confirm the microscopic findings, but over the whole 17 years 5·9 % of all fungi encountered have been incompletely identified

because the cultures failed to grow or were pleomorphic. There are three reasons for this. The main, and fairly constant inhibiting factor is premedication with some fungicide, but, in the course of the work, two other factors appeared, both of which were found to be remediable. In the first year of the survey 20 % of the

Map 2. Birmingham postal districts (including Smethwick, Staffs.). Reproduced by courtesy of H.M. Postmaster General.

Microspora could not be identified exactly on account of the unsatisfactory culture-medium (pure maltose-agar) in use here at the time. When the standard American medium of Hodges (1928) was adopted at the beginning of 1946, followed in June of the same year by the Oxoid medium (Carlier, 1948) still in use, this figure fell to 5 %. Between 1947 and 1949 frequent staff changes adversely

affected the quality of media made from chemically satisfactory ingredients, so that incomplete identifications fluctuated between 11 and 16% in these 3 years. On the appointment of a keen and reliable young technician, this figure dropped at once to 5·7%, falling to an average of 2·3% as she and her assistant gained the experience by which their successors still profit. The present low rate of culture failures of recognizable ringworm fungi (0·6%) is thus attributable to premedication. In addition, there has always been a proportion of specimens which are barely recognizable by microscopy as fungal material at all. They are dead before the patient arrives. It is impossible to say whether the hyphal remnants and shrivelled spores found in the skin samples are those of pathogens or of contaminants. Since 1953, 2·6% of all specimens handled have fallen into this category.

Special media, e.g. thiamine-glucose-agar, are used (as necessary) to accelerate the development of slow-growing and dysgonic fungi (Walker, 1950*b*). Others, of which cornmeal agar, reinforced with glucose is a useful example, serve to discriminate between organisms such as *Trichophyton mentagrophytes* and *T. rubrum* that are liable to be confused on standard proof medium. When the use of such media is indicated, a provisional report is sent to the dermatologist concerned, so that treatment need not be delayed in the interests of botanical precision.

OBSERVATIONS AND DISCUSSION

In the period under review, 1507 cases of ringworm have been referred from all parts of the city. Of these, 1419 were fully identifiable. The remaining 88 were recognized as 74 Microspora and 14 Trichophyta of indeterminate species (Table 1). Of the three genera involved the Microspora are by far the commonest and *Epidermophyton* the rarest (Fig. 1).

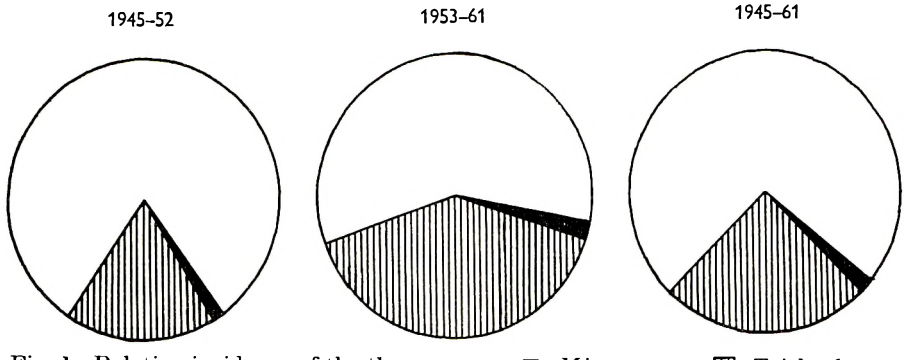


Fig. 1. Relative incidence of the three genera. □, *Microsporum*; ▥, *Trichophyton*; ■, *Epidermophyton*.

Microspora

The geographical distribution of the three species of *Microsporum* is shown in detail in Table 2.

Apart from a minor outbreak of *M. audouini* in an orphanage at Handsworth (Birmingham 20) in 1946, the cases were sporadic and scattered in small numbers

Table 1. *Annual specific incidence*

Species	1945	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	Total
Microsporum audouini	41	78	49	26	13	22	11	13	5	7	10	6	4	1	4	1	1	292
Microsporum canis	19	53	41	01	74	71	88	71	53	32	37	33	31	23	9	3	7	736
Microsporum gypseum	.	1	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	2
Microsporum unspecified	15	8	6	14	7	6	3	2	4	4	3	1	.	.	1	.	.	74
Trichophyton mentagrophytes	8	14	10	19	19	14	17	20	18	18	12	7	11	11	13	7	2	220
Trichophyton tonsurans	2	2	2	2	4	5	4	7	4	3	17	8	11	1	5	3	4	84
Trichophyton rubrum	.	.	.	1	2	.	.	3	1	1	1	1	3	1	.	4	.	18
Trichophyton schoenleini	1	.	.	1	2	1	.	1	1	3	5	.	.	.	.	.	.	15
Trichophyton violaceum	.	2	.	1	.	.	.	.	.	.	.	.	1	.	1	.	.	5
Trichophyton verrucosum	.	.	1	1	1	2	.	2	2	.	3	2	1	2	2	2	1	22
Trichophyton unspecified	1	.	2	4	3	1	.	1	2	.	.	.	.	.	.	.	.	14
Epidermophyton floccosum	1	3	3	1	1	1	1	3	2	2	.	.	2	1	.	3	1	25
	88	161	114	161	126	123	124	123	93	70	88	58	64	40	35	23	16	1507

Table 2. *Distribution of the* Microspora *over the city of Birmingham*

Postal district	*Microsporum audouini* 1945	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	*Microsporum canis* 45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	*Microsporum* unspecified 45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	Total
1	.	.	.	1	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	2	1	1	1	2	.	1	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	14
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—
3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	i	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
4	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2	2	5	1	3	.	.	.	1	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	.	.	.	.	.	.	19
5	.	1	1	.	.	.	.	1	.	.	.	1	.	.	.	.	.	1	1	3	1	4	.	1	3	3	.	.	.	.	1	1	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	25
6	3	4	1	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2	7	7	4	2	1	1	.	9	.	.	1	.	1	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	49
7	2	3	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	2	3	1	7	3	2	6	5	2	1	.	.	3	2	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	46
8	2	2	.	.	.	.	.	.	1	1	1	.	.	.	.	.	.	.	.	.	2	2	4	4	3	1	1	3	5	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	33
9	.	1	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2	5	1	.	.	2	1	4	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	22
10	2	1	1	1	.	2	.	.	.	1	1	1	.	.	.	.	.	.	2	3	4	6	1	9	8	9	6	6	4	2	2	.	.	1	.	.	3	.	.	.	.	1	.	.	.	.	.	.	.	.	.	77
11	.	2	6	4	.	1	1	2	.	.	.	.	.	.	.	.	.	1	4	6	13	5	2	5	3	1	4	5	.	.	.	.	.	.	.	1	4	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	73
12	5	2	6	4	.	.	3	1	1	.	.	.	.	.	.	.	.	1	5	6	4	12	7	3	2	7	3	.	1	6	9	.	1	.	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	93
13	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	1	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6
14	2	2	.	1	.	.	.	1	2	.	.	.	.	.	.	.	.	1	2	1	14	7	.	.	.	1	3	2	1	1	1	2	.	.	.	.	2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	47
15	5	.	1	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1	2	.	3	1	9	2	2	1	.	.	.	1	.	.	.	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	31
16	.	3	1	1	.	.	1	.	.	.	.	1	.	.	.	.	.	.	1	.	.	3	1	4	2	2	.	.	2	.	.	.	.	.	1	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	25
17	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	5	1	1	.	3	1	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	16
18	1	.	1	1	2	1	1	2	.	.	.	.	.	.	1	.	.	.	3	4	12	4	3	4	6	2	1	3	.	.	1	.	.	.	1	.	.	1	.	.	.	1	2	.	.	.	.	.	.	.	.	58
19	.	6	1	.	1	6	.	1	.	.	.	.	.	.	.	.	.	4	2	3	4	1	4	7	5	.	.	2	.	1	2	.	1	2	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	54
20	1	26	6	2	.	.	.	.	1	.	1	.	.	.	.	.	.	.	3	.	1	1	1	3	1	.	.	.	.	.	.	.	.	.	.	1	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	51
21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	1	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
22*a*	.	1	.	1	.	3	.	.	.	.	1	2	.	.	.	.	.	.	3	.	.	1	3	5	4	2	2	1	1	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	31
22*b*	.	.	1	.	.	.	.	.	.	1	.	.	.	.	.	.	1	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	1	.	.	.	.	.	.	8
22*c*	1	4	4	5	8	1	1	1	.	4	6	1	4	.	.	.	.	2	1	4	3	.	1	3	4	.	.	6	1	.	.	1	.	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	68
23	2	.	.	1	.	2	.	.	.	.	.	.	.	.	.	.	.	.	7	.	5	.	2	7	5	2	.	2	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	37
24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
25	1	.	2	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	3	1	2	1	.	3	.	1	.	.	.	.	1	.	.	.	1*	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	18
26	5	2	2	1	.	.	.	2	.	.	.	.	.	3	1	.	.	.	3	1	.	4	1	3	2	2	.	.	1	3	.	.	.	.	3	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	41
27	.	4	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	2	4	3	1	4	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	25
28	.	1	.	.	.	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	2	1	.	.	2	.	.	.	.	2	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	15
29	2	2	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	1	3	.	.	2	1	.	.	.	.	.	.	.	.	.	1	.	.	.	1	1	.	.	1*	.	.	1	.	.	.	.	.	22
30	3	5	1	.	.	1	1	.	.	.	.	.	.	.	.	.	.	3	3	1	5	1	1	1	.	.	.	.	.	1	1	.	.	1	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	32
31	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	1	.	1	3	.	2	1	3	.	.	2	.	.	.	1	1	.	.	.	1	.	.	2	.	.	.	.	.	.	.	.	21
32	.	1	3	1	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	2	.	.	.	1	2	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	14
33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	3	2	2	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10
34	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
P.P.†	3	3	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	14
Total	41	78	49	26	13	22	11	13	5	7	10	6	4	1	4	1	1	19	53	41	91	74	71	88	71	53	32	37	33	31	9	3	7	15	9	6	14	7	6	3	2	5	4	3	1	0	0	1	0	0	0	1104
	292																	736																	74 + 2 (gypseum)																	

* Gypseum. † Private patients, address undisclosed.

over most of the city. At no time during the survey has the incidence of this species approached epidemic proportions in Birmingham. Adamson, in 1895, considered that 80–90 % of all ringworm cases in England were due to *M. audouini*. Indeed, of the 232 cases of *tinea capitis* referred to this hospital from Cheltenham between 1945 and 1948, 90 % were in fact identified as *M. audouini*. Following World War II numerous outbreaks of scalp ringworm among children were reported from all over Great Britain and Ulster. The literature of the subject was thoroughly reviewed by Walker (1958). Many such outbreaks, especially those in remote rural districts, must have been due to initial contact with evacuees from centres of infection. In an East Anglian village, for example, Whittle (1956) found a 'carrier' who had harboured *M. audouini* for two years. At about the same time major epidemics involving 565 children in Hagerstown, Md. (Schwartz *et al.* 1946), some 6000 in Detroit, Michigan (Carrick, 1946) and over 800 in St Paul, Minnesota (Steves & Lynch, 1947) were reported from the U.S.A.

Birmingham, which was systematically bombed every night for a year, so far from being a catchment area, evacuated as many children as possible. This undoubtedly explains the low and declining incidence of *M. audouini* at a time when much of the world was experiencing a recrudescence of a parasite which had been well controlled for the quarter-century before the war. It is interesting to find that within the city boundary the highest average incidence up to 1957 should occur at Kingstanding (Birmingham 22[c]), one of the oldest redevelopment zones. This reflects the influx of children from slum areas during the 'thirties'. The highest actual local concentration of patients amounted to 26 at Handsworth (Birmingham 20) in 1946, and this little outbreak never got out of control. The over-all incidence has gradually declined to one case per annum in the last 2 years.

In Fig. 2 the incidence of *M. audouini* is compared with that of *M. canis* at 6-monthly intervals for the whole 17 years. The cases, especially those caused by *M. canis*, are not confined to *tinea capitis* but comprise all lesions traced to the genus.

Until the introduction of griseofulvin (Gentles, 1958) *tinea capitis* was the occasion of prolonged absence from school in this country. In the U.S.A., where schoolchildren are not segregated for ringworm of the scalp, major epidemics have occurred. Neither misfortune need befall the modern child. The topographical distribution of *M. audouini* and *M. canis* present rather different pictures. *M. canis* which was even more ubiquitous than *M. audouini*, only one postal district being exempt, occurred frequently, though never attaining epidemic proportions, in three adjoining slum and industrial areas, Birmingham 10, 11 and 12, with 62, 49 and 67 cases respectively. Even here the cases must be regarded as essentially sporadic (see Table 2). The northern and central slum region, Birmingham 6, 7, 18 and 19, taken as a unit, constituted another focus of *M. canis* infection, with an aggregate of 157 cases.

The spectacular increase from nineteen cases of *M. canis* over the whole city in 1945 to ninety-one in 1948 was fully described in the earlier survey. The subsequent decline could hardly have been predicted at the time, because it appeared that the steep fall in incidence during the summer of 1952 might be counteracted by an equally steep rise before the end of the year, which proved to be the case. Fig. 3,

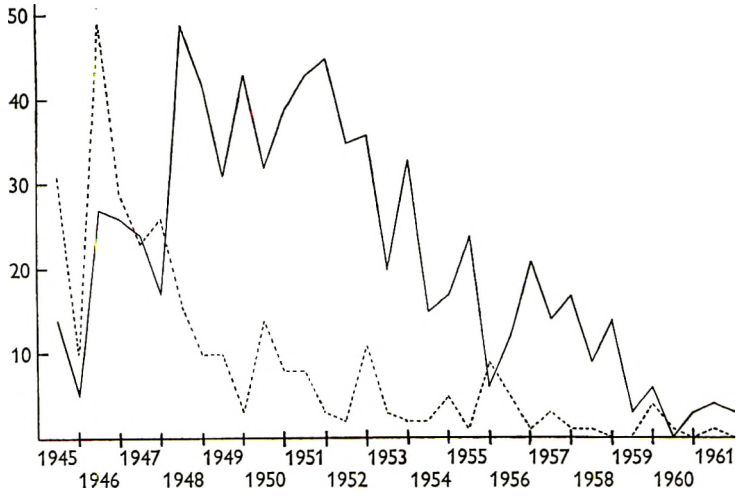


Fig. 2. Sporadic cases of small-spored ringworm, including the Handsworth outbreak, based on the 6-monthly figures, estimated on 30 June and 31 December in each year. The broken line represents *M. audouini* and the entire line *M. canis*.

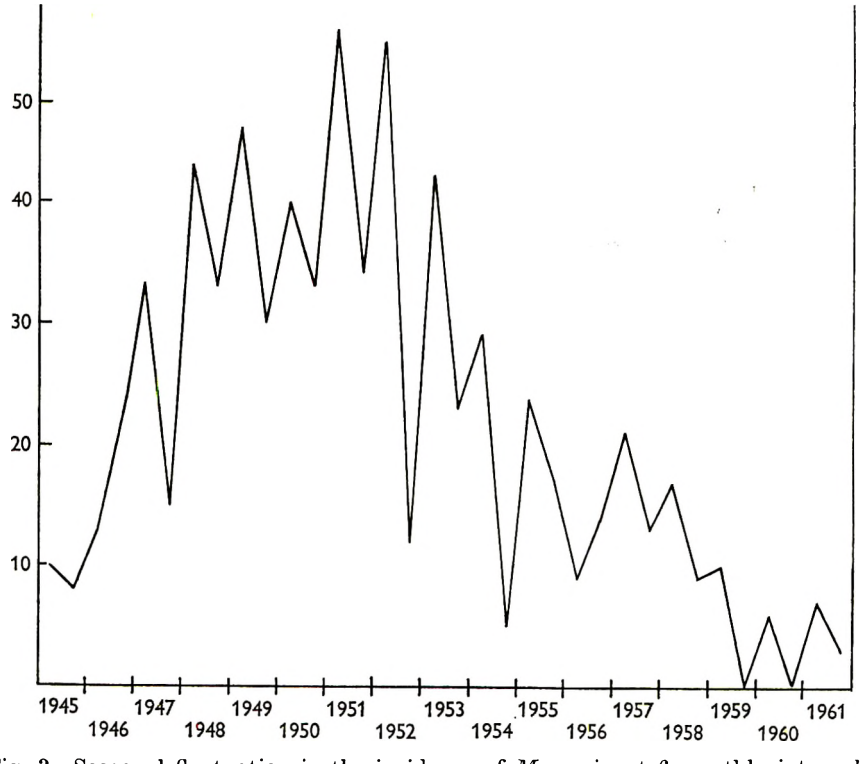


Fig. 3. Seasonal fluctuation in the incidence of *M. canis*, at 6-monthly intervals, calculated on 31 March and 30 September.

which records the seasonal fluctuation in *M. canis*, makes the course of the outbreak clear. From September 1945 to March 1947 the incidence of the species rose continuously, the normal summer trough having failed to appear in 1946, probably due to the severe rationing in force at the time. Naturally the hospital figures cover human cases only. Nothing is known of the incidence among cats and dogs, but it may be assumed with some confidence that domestic pets, relying largely on kitchen scraps for their food, were underfed at this time and probably shewed reduced resistance to the infection. By the end of 1958 the outbreak was over, i.e. before griseofulvin came into general use.

It has been observed elsewhere that from 70 to 80 % of cases of ringworm due to *M. canis* are transmitted from child to child (Walker, 1950*a*). According to Cruickshank (1953) such infections can pass from one child to a succession of others, becoming attenuated in the process and dying out after four to six transfers. In New Zealand, where *M. canis* is rife, Marples (1956) found only 13·9 % of the infected families could trace their infections definitely to a human source, with the probability of a further 7·9 %. She obtained evidence of the following types of transmission: cat to child, dog to child, child to child, child to adult, cat to cat, child to cat, dog to cat and cat to dog.

In Birmingham there is little direct evidence as to how the infection has been contracted, because few pets are brought to the laboratory for examination. However, for four years from 1953 the number of patients was correlated with the number of households involved with the following result:

1953 54 patients from 53 households.
1954 32 patients from 27 households.
1955 31 patients from 27 households.
1956 31 patients from 23 households.

This, together with the geographical scatter, seems to indicate that most of the human cases have been derived direct from animal sources. Two instances, in which child-to-child infection occurred months after the destruction of the kitten concerned, were proved. In other cases, where all the members of a family have had access to the same infected pet, it is impossible to judge whether child-to-child infection actually occurred. In one instance two children from Nechells (Birmingham 7) and their cousin from Aston (Birmingham 6) were known to have played with the same cat, which thus infected two households a mile or so apart.

For many years the two important reservoirs of *M. canis* persisted. What finally led to their elimination is not easy to determine. No statistical relationship can be traced between the changing population figures occasioned by slum clearance and the disappearance of the fungus. Demolition of outworn buildings—however desirable from many points of view—can have no direct effect on such an organism as *M. canis*, which normally disseminates by short-lived and not by resting spores. More probably the extirpation of individual cats, whether ordered by a dermatologist or considered expedient by the owner on moving house, is the explanation. At its worst, the problem was a small-scale one. A single popular kitten—or a pathetic little stray—can infect a lot of children in the close proximity of slum

conditions. What is surprising is that the highly mobile human population failed to spread the infection by public transport and that even in the peak years no epidemic ever developed.

In culture four variants of *M. canis* appeared. Thirty-eight specimens were of the citreous and three of the non-pigmented (*M. canis*, var. *album*) type, described by Walker (1950*b*), five were pleomorphic and the rest produced typical normal colonies.

M. gypseum is rare in Birmingham. Only two cases have occurred several miles apart at an interval of 7 years. No information could be elicited about the first case. The second was traced to the patient's dog. As *M. gypseum* is known to lie dormant in the soil, samples of garden soil collected from about half the postal districts of Birmingham were 'baited' with human and horse-hair by the method of Vanbreuseghem (1952) in an attempt to ascertain if there is much of it in the saprophytic state in this part of the world. Though another keratinophilic fungus, *Keratinomyces ajelloi*, was found in every sample, no single instance of *M. gypseum* appeared and the experiment was abandoned for lack of time.

Trichophyta

Of all the Trichophyta the small-spored *Trichophyton mentagrophytes* is much the commonest. It occurs throughout the city in small numbers, with no discernible focus of concentration. The highest incidence is in the suburbs that constitute Birmingham 14, but the numbers are too small to admit of any generalizations about them. This is a zoophilic fungus with a large variety of known animal vectors, including wild ones, such as rodents and the hedgehog. Two cultures of the hedgehog type with the characteristic golden subsurface pigmentation, have been seen in the laboratory and remembered, but their origin went unrecognized until an exact description was published by English, Evans, Hewitt & Warin (1962). They were included among the *asteroides* type when they were recorded and cannot be extracted at this stage, so their exact location is lost.

Of the 220 *T. mentagrophytes* specimens isolated, 129 were of the *asteroides*, 74 of the *interdigitale* and 17 of the *niveum* types. The last is the cat *Trichophyton* of Sabouraud (1910) and in several of the local instances was actually traced to cats.

Patients of both sexes and all ages have appeared with ringworm due to this species of fungus. The sites affected are the scalp, the beard region, the glabrous skin and the nails. *T. mentagrophytes* var. *interdigitale* is the commonest cause of 'athlete's foot' in this locality.

T. tonsurans was a rare species here until the local outbreak that began in 1954 and lasted for 4 years. Thirty-nine cases, of which eighteen came from Monyhull colony (Birmingham 14), were referred from the city, mainly from the outer suburbs. Within this period seven patients from Staffordshire, five from Warwickshire and one from Worcestershire were seen with the same infection. The scalp was attacked in ten cases, all children; the nails in four, all adults; and the smooth skin alone in the others.

Only five of the thirteen varieties listed by Sabouraud (1910) as separate species, including the four recognized by the M.R.C. (1949), occurred in primary culture.

Table 3. *Distribution of the* Trichophyta *and* Epidermophyton

Postal district	*Trichophyton Mentagrophytes*																	Other Trichophyta																*Epidermophyton floccosum*																Total
	1945	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	
1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1	.	.	.	.	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
2	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
3	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
4	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
5	.	.	.	.	1	2	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	1	.	2	.	.	2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10
6	.	.	.	1	2	2	1	.	.	1	.	.	.	.	1	.	.	.	.	.	2	.	.	.	1	.	.	1	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	13
7	1	.	.	.	.	1	.	1	2	1	.	.	.	2	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1	.	.	13
8	1	.	2	.	1	.	1	2	1	.	1	.	2	1	1	1	.	.	1	2	1	.	.	3	.	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	25
9	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
10	.	.	.	1	.	.	.	.	2	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	7
11	1	1	1	.	1	1	1	.	.	1	5	1	.	.	.	.	.	.	1	.	1	1	.	.	2	.	2	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	22
12	1	2	.	1	.	3	.	2	.	1	.	.	1	.	1	.	1	.	.	.	.	1	1	.	.	.	1	1	1	.	.	1	.	1	.	1	.	1	.	.	.	.	1	.	.	.	.	.	.	.	.	22
13	.	.	.	.	.	.	.	.	1	.	1	1	.	1	.	1	1	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
14	.	3	1	2	2	1	3	3	1	.	.	1	2	.	.	.	.	.	.	.	1	.	1	1	2	.	1	11	4	1	2	1	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	46
15	.	.	.	1	1	.	1	.	.	1	.	.	1	.	.	.	.	.	.	.	2	.	.	.	1	.	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	11
16	.	.	.	1	1	1	.	.	1	1	.	.	.	1	.	1	.	.	.	.	1	.	1	.	.	2	.	.	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	13
17	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
18	1	1	.	1	1	1	1	2	2	.	.	.	1	.	5	1	.	1	.	.	.	3	.	.	.	.	.	.	.	2	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	24
19	.	.	1	1	.	.	1	2	1	1	.	.	.	.	.	.	.	.	.	.	.	1	2	.	.	.	3	3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	18
20	.	.	1	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	.	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	8
21	.	1	.	.	.	.	.	1	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
22*a*	.	.	.	2	.	.	2	.	2	1	.	.	.	2	1	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	13
22*b*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
22*c*	.	2	.	1	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	10
23	1	.	.	2	2	.	2	1	1	1	1	.	.	.	.	1	.	1	1	.	.	1	.	.	.	1	.	.	.	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	19
24	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
25	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
26	1	1	1	.	2	.	.	1	1	1	1	.	.	.	2	.	.	1	.	.	1	.	.	1	1	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	17
27	.	.	1	1	.	1	.	1	.	.	.	.	.	.	1	1	.	.	.	.	1	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	1	.	.	.	1	.	.	.	1	.	.	.	11
28	.	.	1	1	1	.	.	.	1	1	.	1	1	1	.	.	.	.	.	1	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10
29	.	1	.	.	.	.	1	1	1	1	.	.	.	.	.	.	.	.	.	1	.	.	2	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	11
30	.	.	.	1	1	1	1	.	1	.	1	1	.	.	.	.	.	.	.	.	.	1	.	.	1	.	1	1	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	14
31	1	2	1	.	1	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	13
32	.	.	.	1	1	.	.	.	.	.	.	.	2	1	.	.	.	.	.	.	1	.	.	.	.	.	1	1	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	11
33	.	.	.	.	.	.	.	.	.	1	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	4
34	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
P.P.*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
Total	8	14	10	19	19	14	17	20	18	18	12	7	11	11	13	7	2	4	4	5	10	12	9	4	14	10	7	26	11	16	4	8	9	5	1	3	3	1	1	1	1	3	2	2	0	0	2	1	0	3	1	408
	220																	158																	25																	

* Private patients, address undisclosed.

In the seventeen years of the survey the Birmingham incidence is as follows: *T. tonsurans*, var. *flavum*, 35 cases; var. *sabouraudi*, 17 cases; var. *sulphureum*, 8 cases; var. *tonsurans*, 5 cases; var. *plicatile*, 7 cases.

T. rubrum, though known to be on the increase in many parts of the country is still a rarity here. According to Walker (1950*a*) this species, 'a native of the Far East, is increasing in Britain, aided by importations from abroad chiefly from South-East Asia'. She cites three cases known to have originated in India out of seventeen proved importations. In all, only eighteen cases have been diagnosed at this hospital. There is no apparent connexion between the low annual incidence shown in Table 1 and the large Indian and Pakistani population at present in the city. If these people had introduced *T. rubrum* it should have become apparent by this time. *Tinea pedis* is the commonest manifestation of this species. Our records show fourteen such cases, one of *T. barbae*, one of *T. unguium* (confined to the finger-nails) and two of *T. cruris*.

Trichophyton schoenleini is even rarer in Birmingham. Nine of the fifteen cases belong to two families. The rest occurred singly, two in institutions. The favus is confined to the scalp in all instances.

T. violaceum is rarer still. In three of the five cases there was a history of importation from Poland, the Far East and Holland. About the other two no information was forthcoming, one being an Arab with no English.

Even in a heavily industrialized city cattle ringworm is not unknown. Slaughtermen are liable to it and children sometimes bring it back from country holidays. *T. verrucosum* at the rate of about 1·5 cases a year can hardly be considered a serious menace, though some of the lesions are severe with painful kerions. Half the cases seen involved the hair of either the scalp or the beard region. *T. verrucosum* var. *discoides* is the usual colonial type here, var. *ochraceum* having been seen three times and var. *album* once.

Epidermophyton

Epidermophyton floccosum occurs singly. Ten of the patients showed groin involvement, two *tinea pedis* and the rest lesions of the limbs, axilla and neck. No case of nail-ringworm due to *E. floccosum* has been treated here. Most of the patients were ex-service personnel and their contacts.

CONCLUSIONS

It is of some interest to compare the incidence of species peculiar to man with those common to man and one or more of the lower animals.

Zoophilic fungi are:

Microsporum canis	*Trichophyton mentagrophytes*
M. gypseum	*T. verrucosum*

There is some uncertainty about whether *M. gypseum* should be included under this heading because, although it is known to infect a variety of animals, wild and domestic, there is evidence that it can attack man without any animal host as

intermediary (Ajello, 1953; Whittle, 1956). In any case, our two instances are of small statistical significance.

The anthropophilic species comprise:

Microsporum audouini	*T. schoenleini*
Trichophyton tonsurans	*T. violaceum*
T. rubrum	*Epidermophyton floccosum*

Sabourand (1910) suggested that the *T. flavum* (*cerebriforme* in his nomenclature) variety of *T. tonsurans*, which he regarded as a separate species, might have an animal host, despite his failure to find one. This guess has been disproved in recent years by several mycologists working independently. The monospore culture method of Georg (1957) and the hyphal fusion technique of Davidson, Dowding & Buller (1932) applied in the U.S.A. by Taschdjian & Muskatblit (1955) and in this country by the author, should be enough to dispel the idea that *T. flavum* constitutes a species at all. The whole species, including this variety, may thus be confidently classified as anthropophilic.

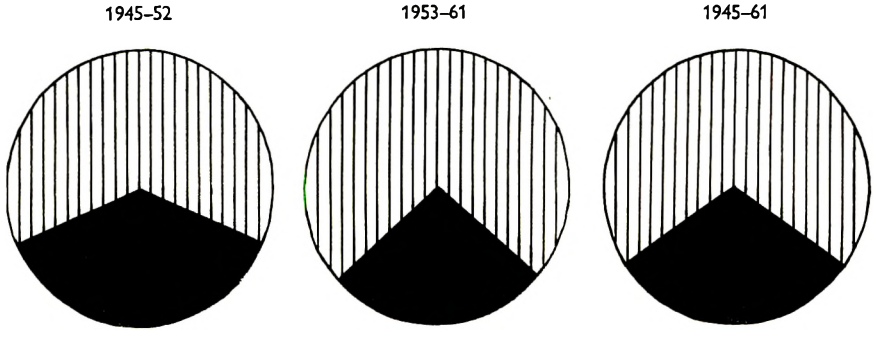


Fig. 4. Relative proportion of zoophilic and anthropophilic species. ▥, Zoophilic fungi; ■, anthropophilic.

Fig. 4 shows the relative proportions of zoophilic and anthrophilic fungi of Birmingham from 1945 to 1952, from 1953 to 1961 and over the entire span of 17 years. Though reflecting the fluctuations described in the foregoing section, all three pie-charts show a marked preponderance of zoophilic species.

This has been observed elsewhere on this island, not only in country districts where it is to be expected but in highly industrialized cities, e.g. Coventry, Leeds, Leicester, Portsmouth and Reading (Walker, 1958). A study of rural and urban ringworm in north-eastern Michigan (Georg, Hand & Menges, 1956) revealed that urban ringworm of animal origin is derived mainly from cats and dogs. The same is true here. Fig. 5 shows the relative incidence of ringworm contracted from dogs and cats compared with that of infections associated with other animals.

The outbreaks of *M. audouini*, *M. canis* and *T. tonsurans* have subsided. There remains a small amount of sporadic fungus infection, consisting mainly of *T. mentagrophytes* and *M. canis*, but how much of this is imported and how much indigenous it would be hard to determine.

In the so-called 'twilight' areas, inhabited to high density by Indian, Pakistani

and West Indian immigrants, who live under conditions that provoke continual comment from the local press, there is no demonstrable increase in the ringworm flora.

It is unlikely that an epidemic of ringworm comparable to the *M. canis* outbreak of 1946–58 will ever occur again in Birmingham. As all the common dermatophytes are controllable by griseofulvin administered orally (Gentles, 1959), it is probable that every case that does occur and is promptly treated will be of such short duration that few contacts will be made and that they, in turn, will be limited by the same antibiotic. The main danger will lie in *T. rubrum* infections of the toenails, which are resistant. Such cases do not appear to be on the increase in this region, however.

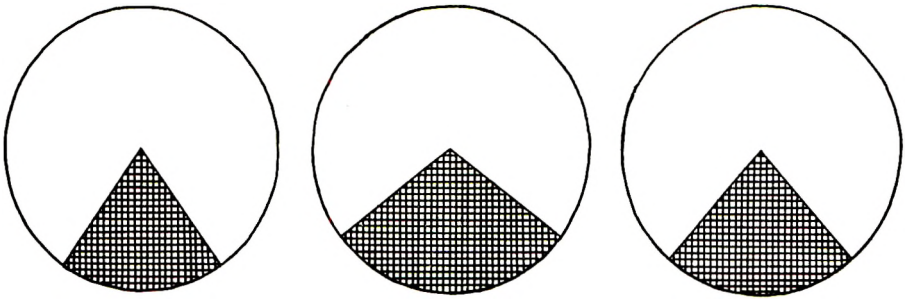


Fig. 5. Animal hosts. □, Cat and dog; ⊞, miscellaneous animals.

Birmingham can no longer be regarded as a centre of much interest to the clinical mycologist, except in so far as fundamental research is concerned; and for that the raw materials are in danger of petering out. For this reason live cultures of the common dermatophytes have been distributed from this laboratory to other hospitals in the vicinity.

SUMMARY

The nature of the ringworm flora of Birmingham over 17 years was investigated and tabulated by species, geographical distribution and concentration.

The rise and fall of such outbreaks as occurred has been charted.

The persistent preponderance of zoophilic species, in spite of the recent decline in over-all incidence, has been demonstrated.

The author is much indebted to Dr Baylis Ash, Dr Avit Scott, the late Dr Cranston Walker, Dr Henly and Dr Hocken Robertson for referring so many interesting cases to this laboratory; to Alderman Sir Albert Bradbeer, Prof. Lancelot Hogben, Mr Alan Neale of the City of Birmingham Central Statistical Office and to Mr W. H. Daley, Allocations Officer to the City of Birmingham Housing Management Department, for municipal statistical material; to the late Dr Dykierman, the late Dr Hirst and Mr Bertram Sealey for the loan of literature; to Sister Simpson and to the Misses G. Hyde, J. Shakespeare, M. A. Lardner, B. Webster, J. Payne and B. Duffill for technical assistance and to Dr Chaplin, Consultant Pathologist to the Group, for certain facilities not available at the Skin Hospital.

REFERENCES

ADAMSON, H. G. (1895). Observations on the parasites of ringworm. *Brit. J. Derm.* **7**, 201.

AJELLO, L. (1953). The dermatophyte, *Microsporum gypseum*, as a saprophyte and parasite. *J. invest. Derm.* **21**, 157.

CARLIER, G. I. M. (1948). An all-British mycological culture medium. *Brit. J. Derm.* **60**, 61.

CARLIER, G. I. M. (1954). An eight-year survey of the ringworm flora of Birmingham. *J. Hyg., Camb.*, **52**, 264.

CARRICK, L. (1946). Methods of local therapy for *Tinea capitis* due to *Microsporon audouini*. *J. Amer. med. Ass.* **131**, 1189.

CRUICKSHANK, R. (1953). Epidemiology of some skin infections. *Brit. med. J.* i, 55.

DAVIDSON, A. M., DOWDING, E. S. & BULLER, A. R. H. (1932). Hyphal fusions in dermatophytes. *Canad. J. Res.* **6**, 1.

ENGLISH, M. P., EVANS, C. D., HEWITT, M. & WARIN, R. P. (1962). 'Hedgehog ringworm'. *Brit. med. J.* i, 149.

GENTLES, J. C. (1958). Experimental ringworm in guinea pigs: oral treatment with griseofulvin. *Nature, Lond.*, **182**, 476.

GENTLES, J. C. (1959). The treatment of ringworm with griseofulvin. *Brit. J. Derm.* **71**, 427.

GEORG, L. K. (1957). *Dermatophytes. New Methods in Classification.* Atlanta, Ga.: U.S. Department of Health, Education and Welfare.

GEORG, L. K., AJELLO, L. & PAPAGEORGE, C. (1954). Use of cycloheximide in the selective isolation of fungi pathogenic to man. *J. Lab. clin. Med.* **44**, 422.

GEORG, L. K., HAND, E. A. & MENGES, R. A. (1956). Observations on rural and urban ringworm. *J. invest. Derm.* **27**, 335.

HODGES, R. S. (1928). Cultures of ringworm fungus on Sabouraud proof mediums and on mediums prepared with American peptones and sugars: comparative study. *Arch. Derm. Syph., N.Y.*, **18**, 852.

MARPLES, M. J. (1956). The ecology of *Microsporum canis* Bodin in New Zealand. *J. Hyg., Camb.*, **54**, 378.

M.R.C. MEMORANDUM, No. 23 (1949). *Nomenclature of Fungi Pathogenic to Man and Animals.* H.M. Stationery Office.

M.R.C. MEMORANDUM, no. 23, revised edn. (1958). *Nomenclature of Fungi Pathogenic to Man and Animals.* H.M. Stationery Office.

SABOURAUD, R. (1910). *Les Teignes.* Paris: Masson.

SCHWARTZ, L., PECK, S. M., BOTVINICK, I., LEIBOVITZ, A. L. & FRAZIER, E. S. (1946). Control of ringworm of the scalp among school children in Hagerstown, Md., 1944–5. *J. Amer. med. Ass.* **132**, 58.

STEVES, R. J. & LYNCH, F. W. (1947). Ringworm of the scalp. Report of the present epidemic. *J. Amer. med. Ass.* **133**, 306.

TASCHDJIAN, C. & MUSKATBLIT, E. (1955). Hyphal fusion between *Trichophyton tonsurans* variants as an indication of species relationships. *Mycologia*, **47**, 339.

VANBREUSEGHEM, R. (1952). Technique biologique pour l'isolement des dermatophytes du sol. *Ann. Soc. belge Méd. trop.* **32**, 173.

WALKER, J. (1950*a*). The dermatophytoses of Great Britain. Report of a three years' survey. *Brit. J. Derm.* **62**, 239.

WALKER, J. (1950*b*). Variation in *Microsporum canis* and *Microsporum audouini*. *Brit. J. Derm.* **62**, 395.

WALKER, J. (1958). Survey of the medical mycological literature of Great Britain 1946 to 1956. *Mycopathologia*, **9**, 307.

WHITTLE, G. H. (1956). A survey of fungous infection in the Cambridge area 1948–1955. *Brit. J. Derm.* **68**, 1.

J. Hyg., Camb. (1963), **61**, 307
With 1 *plate*
Printed in Great Britain

Autoradiographic detection of radioactive bacteria introduced into sea water and sewage

BY MATHS BERLIN AND RAGNAR RYLANDER

From the Institute of Hygiene, Karolinska Institute and the National Institute of Public Health, Department of General Hygiene, Stockholm, Sweden

(*Received* 1 *March* 1963)

The agency of water in the dissemination of bacteria and viruses has long been recognized, but though measures have been taken to control this hazard, water still plays a large role in spreading pathogens such as enterovirus and salmonella. A simple and reliable method for studying dissemination of this kind would therefore be most useful.

The presence in water of thermostable coli bacteria is generally considered an index of fæcal contamination. Unfortunately, their detection in a water sample is a laborious procedure which requires growing cultures, often on different media, for confirmation. Furthermore, the significance of one particular source of contamination when several are present cannot be determined with current bacteriological technique. The problem has been previously attacked by using mutants of the contaminating micro-organisms, or indicator organisms such as *Serratia* (Robson, 1956). The tracer bacteria are added to the water at the source of contamination and water samples are taken for bacteriological examination.

Radioactive substances and dyes have also been used to study the spread of contamination (Montens, 1954; Ely, 1957). Because of rapid dilution, high levels of radioactivity are necessary to follow dispersion in large volumes of water. In addition, the action of biological factors on the survival of contaminating bacteria cannot be ascertained with these materials alone.

Bacteria which have been radioactively labelled can be used as tracer organisms. If they can be detected by a method which avoids the necessity of culturing water samples and if the method is sufficiently sensitive, the use of radioactive bacteria offers several advantages. In the present paper such a method is described, for use in studying the spread of bacteria in water. Water samples were filtered through a Millipore filter and an autoradiogram of the filter was prepared in which the bacteria appeared as black spots. The accuracy and sensitivity of the method were estimated and a test made under field conditions.

METHODS

Strain B *Escherichia coli* were used. The bacteria were grown overnight at 37° C. in agitated 500 ml. flasks on Andersson's synthetic medium.* The medium was modified by replacing $MgSO_4$ with $MgCl_2$ to eliminate carrier sulphur. One micro-

* $MgSO_4$, 0·1 g.; Na_2HPO_4, 6 g.; KH_2PO_4, 3 g.; NaCl, 5 g.; NH_4Cl, 1 g.; glucose, 4 g.; H_2O, ad 1000 ml.; pH = 7.

curie ^{35}S, carrier-free, in 2 ml. isotonic saline solution, was added per 100 ml. medium. This synthetic medium does not provide for optimal growth but the low sulphur content is necessary for the bacterial production of amino acids labelled with radiosulphur. The presence of organic sulphur compounds would prevent the incorporation in the bacteria of radiosulphur in sufficient amounts. A culture containing 3×10^8–5×10^9 bacteria/ml., as determined by direct counting under the microscope, was generally used in the experiments.

The number of bacteria in water samples to which a known number of bacteria had been added was determined both by viable count and by the autoradiographic technique. The water samples were prepared from the bacterial culture by dilution with 0·9 % NaCl to an estimated concentration of 10 (sample B 1) and 100 (sample B 2) bacteria per ml. A control sample (C) was made with sterile radioactive media to the same dilution as B 2.

The viable count was performed by inoculating 1 ml. from each water sample in nutrient agar and incubating at 37° C. for 24 hr.

The autoradiographic determination of bacteria was made by diluting 1 ml. from each water sample in 500 ml. of 0·9 % NaCl and filtering the solution through a Millipore filter (type HA, pore size 0·45 μ). The filters were dried at 50° C. for 20 min. and applied to X-ray film (Gevaert Osray). After 10 days' exposure the films were developed. Black spots (Pl. 1) which appeared similar in structure to autoradiographic images of bacteria obtained by other authors (e.g. Stonier, 1956) were taken to be bacteria. The spots were counted under × 10 magnification, but some films were also counted under higher magnification. The number of spots on unexposed films from the same package as the exposed films was also determined.

Ten determinations were made from all water samples and the autoradiograms from different samples were counted independently of each other so that the technician making the counts was not influenced by previous totals. Stainless steel funnels were used for filtration and were washed twice with 0·9 % NaCl at the end of every filtration to remove any radioactive medium present. Before they were developed, the films were separated from the filters under water to avoid electrostatic phenomena which might cause black spots on the films.

The possibility that a thin layer of sludge on the filter interfered with the number of spots was investigated by filtering water samples obtained by diluting the culture of labelled bacteria with heavily polluted water instead of 0·9 % NaCl.

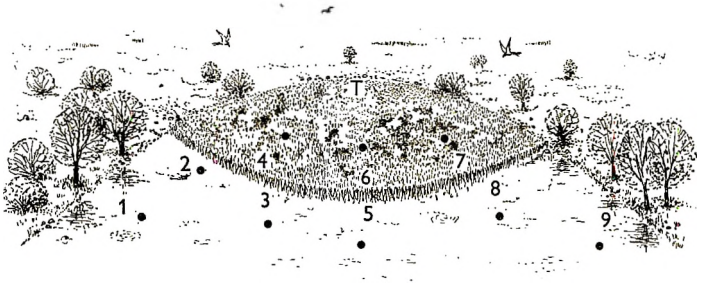
The survival of radioactive bacteria was studied in two water samples. One sample was kept at room temperature and the other at 4° C. Determinations of the number of bacteria were made at regular intervals by using the autoradiographic technique and viable count.

To obtain an idea of the practicability of the autoradiographic method, a study was made of the spread of bacteria from the effluent of a household septic tank which discharged via a sewer into a small inlet of the Baltic Sea near Stockholm. A dense stand of reeds extended for 30–40 m. on both sides of the sewer outlet and in front, for some 20 m. out into water 2 m. deep (Text-fig. 1).

To investigate the presence of substances which might stimulate bacteria and to investigate how this 'background' varied, autoradiograms were prepared from water samples obtained before radioactive bacteria were added to the sewer.

With the aid of a dye a preliminary description of the spread of sewage was obtained, and a number of sites for sampling was selected.

A 200 ml. portion of medium containing radioactive bacteria was then added to the contents of the septic tank by pouring the bacteria into a water closet. For 24 hr. afterwards water samples were taken at varying intervals at the different sampling sites. Two filters were prepared from every sample and the bacteria



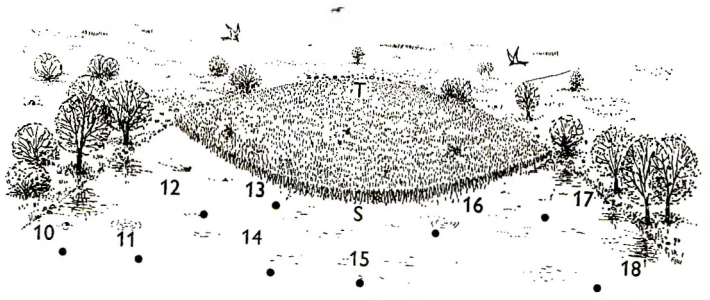
	Site 1		2		3		4		5	
	Film	Film	Film	Film	Film	Film	Film	Film	Film	Film
Hours	1	2	1	2	1	2	1	2	1	2
⅓	—	—	—	—	—	—	123	148	—	—
1½	—	—	—	—	616	835	∞	∞	46	14
4	14	6	1378	1545	∞	∞	∞	∞	5	5
8	55	80	151	160	2344	2459	∞	∞	45	38
18	3	1	3	6	93	112	∞	∞	38	44
24	3	6	4	13	287	277	1029	1097	73	92

	Site 6		T		7		8		9	
	Film	Film	Film	Film	Film	Film	Film	Film	Film	Film
Hours	1	2	1	2	1	2	1	2	1	2
⅓	54	47	∞	∞	30	26	—	—	—	—
1½	482	493	∞	∞	22	27	44	51	—	—
4	∞	∞	∞	∞	3	0	14	15	6	11
8	20	24	∞	∞	7	2	12	19	7	3
18	59	58	∞	∞	6	13	2	0	6	1
24	33	216	∞	∞	9	11	25	44	4	9

Text-fig. 1. Field site, showing sea-shore and stand of reeds (not to scale). The table gives the number of bacteria in autoradiograms from each of ten samples collected at sites indicated, at ⅓, 1½, 4, 8, 18 and 24 hr. after release of radioactive bacteria into septic tank discharging at T. Symbol ∞ indicates bacteria too numerous to count.

appearing in the autoradiograms were counted. Because of windy weather quite a lot of suspended matter was present in the water and only 50 ml. could be used in each filtration.

In an experiment performed some weeks later, 200 ml. of culture containing radioactive bacteria were released beyond the growth of reeds, on the sea bottom. Samples were taken from a depth of ½ m., at varying distances from the outlet (Text-fig. 2). 200 ml. of each water sample were filtered in this experiment.



Site

Hours	10	11	12	13	14	15	S	16	17	18
¼	—	—	—	—	—	10	2924	—	—	—
½	—	—	—	—	12	158	∞	624	—	—
1	—	—	5	15	5	100	∞	∞	∞	—
2	—	7	1869	8	590	2000	∞	∞	∞	1176
8	74	68	7	—	17	10	538	7	24	315
23	9	13	2	19	46	10	11	2	7	4

Text-fig. 2. Number of bacteria in autoradiograms ¼, ½, 1, 2, 8 and 23 hr. after release of radioactive bacteria on sea bottom at site S.

RESULTS

Comparison between viable count and autoradiographic determination

An average of 83 spots (S.D. = 12) and 83 (11) were found in two counts of the autoradiograms prepared from 1 ml. of sample B 1. The number of colonies on the nutrient agar plates from the same sample was 71 (23). Bacterial dilution B 2 gave 612 (44) and 609 (46) spots in two counts of the autoradiograms and 503 (69) colonies on nutrient agar. In the control experiments ten unexposed films showed 1·8 (1·6) black spots. The autoradiograms prepared from 1 ml. of sample C

Table 1

	Bacteria dilution B 1			Bacteria dilution B 2		
		Autoradiography, 1 ml. diluted in 500 ml. NaCl			Autoradiography, 1 ml. diluted in 500 ml., NaCl	
Filtration	Viable count, 1 ml.	Count 1	Count 2	Viable count, 1 ml.	Count 1	Count 2
1	72	102	93	420	644	632
2	87	79	79	428	603	606
3	50	95	95	519	639	655
4	59	80	79	512	604	590
5	71	68	68	563	618	619
6	62	96	100	469	623	614
7	130	81	83	590	671	663
8	70	64	66	403	630	629
9	53	84	83	573	509	497
10	57	79	85	556	582	589
Mean	71·1	82·8	83·1	503·3	612·3	609·4
S.D.	23·4	12·0	11·0	69·0	43·9	46·4

showed 2·3 (1·4) spots. There was no significant difference between the number of spots on unexposed films and films from sample C. Ten nutrient agar plates from sample C contained a total of two colonies. All data are given in Tables 1 and 2.

Table 2. *Number of black spots on unexposed films and on autoradiograms from sterile radioactive medium*

Sample	Unexposed film. Counted spots	Sterile radioactive media C	
		Viable count	Auto-radiography
1	1	0	1
2	1	0	3
3	1	0	2
4	6	0	4
5	1	0	5
6	2	0	1
7	3	0	3
8	1	1	1
9	1	0	2
10	1	1	1
Mean	1·8	—	2·3
S.D.	1·6	—	1·4

Analysis of variance performed on the number of autoradiographic spots showed that with 95 % confidence the population mean lies within ± 26 spots around the sample mean for sample B 1 if only one autoradiogram was counted once and within ± 8 spots if ten films were counted. Recounting did not increase the sensitivity. This was also true of sample B 2 for which 102 spots was the 95 % confidence limit if one film was counted and 32 if ten films were counted.

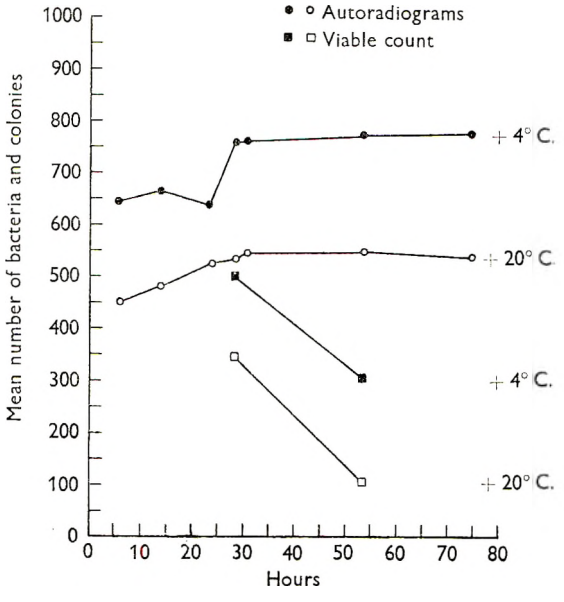
No significant difference in the number of bacteria in the autoradiograms was revealed by comparing the experiments in which 0·9 % NaCl and heavily polluted water were used as diluents.

The results of the survival test show that even after 75 hours storage, the number of bacteria demonstrated in autoradiograms had not decreased but the number of colonies on viable count fell rather rapidly (Text-fig. 3).

Field trials

The number of spots counted on autoradiograms from two natural sea-water samples are given in Table 3. Variance analyses show that the probability of the background exceeding the limit of 12 spots is only 0·1 %. There is a significant difference between the number of spots on autoradiograms from sea water and the number of spots on unexposed films (Tables 2 and 3). This indicates the presence in natural sea water from this source of agents which can produce black spots on the film. Whether these images are the result of radioactivity or of some chemical reaction with the film has not been determined in this experiment.

The data obtained in the field tests are found in Text-figs. 1 and 2. The distribution of bacteria in space and time are consistent with the locations of the sampling sites and the sampling times and also agree well with the fluctuations observed in the dispersion of the dye that was discharged as a control together with the bacteria.



Text-fig. 3. Mean number of bacteria on autoradiograms and colonies on nutrient agar from water samples stored at different temperatures. Each symbol represents the mean of five samples.

Table 3. *Number of spots on autoradiograms from filtrations of natural sea water*

	Sample A		Sample B	
Filtration	Count 1	Count 2	Count 1	Count 2
1	3	3	5	6
2	2	2	6	6
3	3	3	4	6
4	8	8	1	3
5	5	6	10	7
6	6	5	2	2
7	5	6	3	3
8	8	9	3	2
9	3	3	1	1
10	9	7	6	6
Mean	5·2	5·2	4·1	4·2
S.D.	2·5	2·4	2·8	2·2

DISCUSSION

The presence in autoradiograms of images which resemble but are not caused by radioactive bacteria is one factor which limits the sensitivity of the method described here. These images are presumably found in equal numbers in autoradiograms of filters through which water without radioactive bacteria has been filtered. From Table 3 it is evident that the number of 'simulated bacteria' is fairly constant in this experiment. A statistical analysis, performed on a given number of controls, will give probability limits for the number of bacteria that can be detected with the background present. The background value must be determined in every experiment before the radioactive bacteria are added to the water.

If the background spots are examined under greater magnification, some of them show a structure different from that of the bacterial images. These spots can then be excluded and the sensitivity of the method improved (Table 4). Other background spots which cannot be distinguished from bacteria are probably caused by background radiation. The procedure for examining the spots under 80–150 times magnification is rather tedious but under most circumstances such a high sensitivity will probably not be required.

Table 4. *Images identical with those caused by radioactive bacteria, counted on unexposed films under* 10× *and* ×80 *magnification*

Film NR	×10	×80
1	2	1
2	2	1
3	1	0
4	2	1
5	8	6
6	3	0
7	5	3
8	3	0
9	4	1
10	2	1
Mean	3·2	1·4

In practice, a count of only a few bacteria on a filter will be of little consequence —only large counts will be important and these are but little affected by the errors discussed above. It is apparent from the statistical analyses that the sensitivity of the method is limited not by the accuracy of counting the spots on the autoradiograms but rather by the uneven dispersion of bacteria in water.

The amount of radioactivity present in the medium is well below the activity required to cause a decrease in outgrowth (Schmidt, 1948; Rubin, 1954). It is reasonable to assume that the radioactive bacteria do not differ from unmarked bacteria in regard to survival in polluted waters.

A larger number of bacteria are found in the autoradiograms compared to the number of colonies found on viable count. This may be due to the presence of aged bacteria which no longer give colonies on nutrient agar but still retain their

structure and thus give autoradiographic images. It is possible that viable counts on a different kind of medium would yield higher counts. It is also possible that two or more bacteria adhere and give rise to only one colony. This could also explain the larger standard variation of colonies in viable count when compared to the number of autoradiographic bacteria (Table 1). As the time between death and lysis of bacteria is rather short (Koch, 1959) it is unlikely that dead bacteria cause a large part of the difference in number between autoradiographically-demonstrated bacteria and counted colonies. The decrease in the number of colonies on viable count after storage of water samples corresponds to the findings of earlier authors (PHLS Water Committee 1953).

Apparently a thin layer of sludge does not absorb enough radiation from the bacteria to interfere with the autoradiographic results. Because the cells are retained *on* rather than *in* the filter absorption of radiation by the filter is negligible.

The present method appears to be suitable for practical use. A small number of bacteria can be detected and the sensitivity of the method increases if there is less sludge in the water. Samples from heavily polluted water may be centrifuged according to the technique described by Rastgeldi (1959) and autoradiograms prepared from the sediment. This modification of the method, however, remains to be developed.

A great advantage of the method described here is that bacteriological handling is eliminated except for the preparation of the test culture. Suction for filtering can be obtained by whatever means are available, e.g. by manual, motor-powered, or Venturi pumps. The filters should be applied to film immediately but they can be sent by mail if their surfaces are protected. Other bacteria such as enterococci can also be marked with radiosulphur and used in tracer experiments.

If large numbers of bacteria are needed, the sulphur content of the medium can be increased by adding carrier sulphur. At concentrations above 0·005 mg. sulphur/ml. medium, however, the uptake of labelled sulphur diminishes (Cowie, Bolton & Sands, 1952) and less favourable conditions for autoradiography occur. A continuous culture apparatus might also be set up near a river or any other location where a great number of bacteria would be needed to perform the test.

SUMMARY

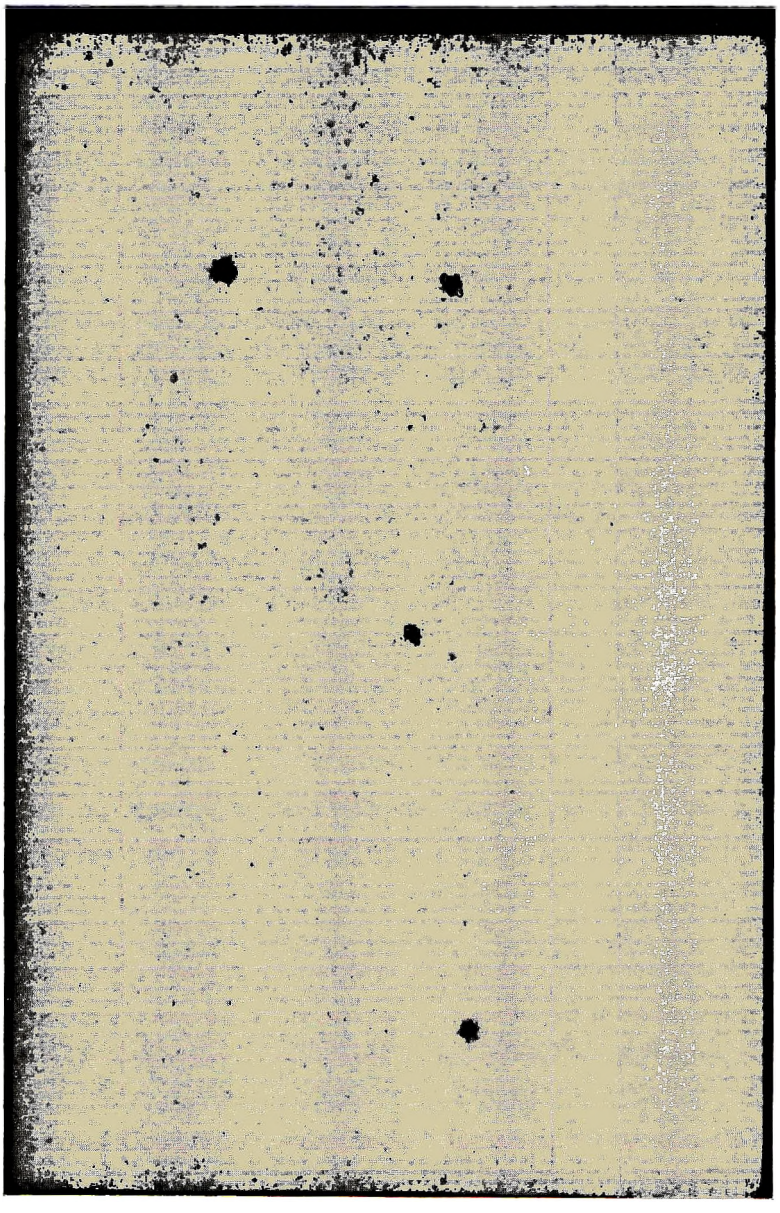
An autoradiographic method to trace the dissemination in water of radioactive bacteria is reported. It has been tested for accuracy and sensitivity and a field trial has been performed. The method appears suitable for practical use and permits the study of environmental effects on the spreading bacteria.

REFERENCES

COWIE, D. B., BOLTON, E. T. & SANDS, M. K. (1952). The labelling of bacterial cells with S^{35} for the production of high specific activity compounds. *Arch. Biochem.* **35**, 140.

ELY, R. L. (1957). Radioactive tracer study of sewage field in Santa Monica Bay. *Ire Trans. Nucl. Sci.*, N.S., **4**, 49.

KOCH, A. (1959). Death of bacteria in growing culture. *J. Bact.* **77**, 623.



MONTENS, A. (1954). Use of radioactive isotopes for water flow and velocity measurements. *Proc. 2nd Radioisotope Conf., Oxford*, pp. 169–80.

PHLS WATER SUB-COMMITTEE (1953). Storage of water samples. *J. Hyg., Camb.*, **51**, 559.

RASTGELDI, S. (1959). The simultaneous separation and concentration of corpuscular elements and bacteria from sputum. *Acta Cytol.* **3**, no. 2, 183.

ROBSON, J. (1956). A bacterial method for tracing sewage pollution. *J. appl. Bact.* **19**, 243.

RUBIN, B. A. (1954). Growth and mutation of bacteria during continuous irradiation. *J. Bact.* **67**, 361.

SCHMIDT, C. F. (1948). The effect of radioactive phosphorus upon a suspension of *Escherichia coli*. *J. Bact.* **55**, 705.

STONIER, T. (1956). Labeling crown gall bacteria with P^{35} for autoradiography. *J. Bact.* **72**, 259.

EXPLANATION OF PLATE

PLATE 1

Detail of an autoradiogram of Millipore filter through which a suspension of radioactive bacteria was passed. Black spots represent bacteria. × 80.

J. Hyg., Camb. (1963), **61**, 317
With 3 plates
Printed in Great Britain

On the recognition and therapy of Simian woolsorter's disease*

By W. S. GOCHENOUR, Jr.,† W. D. SAWYER, J. E. HENDERSON,‡
C. A. GLEISER,§ R. W. KUEHNE and W. D. TIGERTT‡
United States Army Medical Unit, Fort Detrick, Maryland

(*Received* 4 *March* 1963)

In the Brown Lectures of 1880 and 1881 Prof. W. S. Greenfield (1880, 1881), reported the anthrax bacillus to be the aetiological agent of woolsorter's disease and presented what remains today the classical description of the clinical manifestations and morbid anatomy of respiratory anthrax in man. Though at present infrequent, woolsorter's disease still, in the era of antibiotics, is associated with a high mortality rate. Experimental evaluation of therapeutic approaches to woolsorter's disease, as distinguished from cutaneous anthrax, has been limited since the mediastinal cellulitis and intrathoracic lymphadenitis characteristic of the human disease have not been observed in experimental respiratory anthrax of lower animals. For example, guinea-pigs, sheep and rhesus monkeys develop a septicaemia, and such lesions as are found at post-mortem examination are secondary to this septicaemia. Recent observations (Gochenour, Gleiser & Tigertt, 1962) of the clinical course and morbid anatomy in rhesus monkeys receiving early, inadequate penicillin prophylaxis following inhalation of spores of *Bacillus anthracis* indicated that some animals so modified had a febrile course and exhibited extensive intrathoracic lymph node and mediastinal involvement. Such animals closely simulate the pattern of woolsorter's disease as originally described so clearly by Greenfield and confirmed by others. These animals have served as a model for examination of therapy with penicillin and with tetracycline.

MATERIALS AND METHODS

The anthrax spore suspension and respiratory exposure were as previously described by Gochenour and his associates. Thirty-eight young adult *Macaca mulatta*, weighing from 2·3 to 3·1 kg., were exposed to inhaled doses of spores ranging from 7000 to 1,016,000 with a mean of 621,000. Beginning 24 hr. after respiratory exposure all received 150,000 units of procaine penicillin G intramuscularly once daily for 5 days. Conventional (65 kV, 30 MA.) and supervoltage (1 MeV., 3 MA.) chest roentgenograms were obtained on all animals before respiratory exposure and at 12 hr. intervals after discontinuance of penicillin. In

* The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed in this study.

Present addresses:
† Deseret Test Center, Salt Lake City, Utah.
‡ Walter Reed Army Institute of Research, Washington, D.C.
§ Army Mission to Panama, Box 2016, Balboa, Canal Zone, % U.S. Embassy.

addition, rectal temperatures and clinical evaluation were accomplished at 6 or 8 hr. intervals. In some animals blood cultures were obtained at the time the second course of treatment was to begin.

In these studies limited animal-holding facilities and the extent of observations upon individual animals necessitated four separate exposures and study periods. Since presented doses of spores and test subjects and their responses appeared comparable they have been considered together.

Upon clear evidence of chest involvement therapy was instituted (exclusive of controls) either with penicillin (300,000 units procaine penicillin G intramuscularly at 8 or at 12 hr. intervals for 7 days) or tetracycline (100 mg. intramuscularly every 6 hr. for 5 days then every 12 hr. for an additional 5 days; two monkeys received 100 mg. intramuscularly and 125 mg. intragastrically followed by 125 mg. intragastrically every 8 hr. for 7 days). Aside from considerable myositis due to intramuscular tetracycline, the factor causing the change to intragastric administration, therapy *per se* was uncomplicated.

Complete post-mortem examinations were performed on all animals. Those surviving the acute phase of the study were killed between 55 and 84 days following exposure.

RESULTS

Early penicillin prophylaxis. As previously reported (Gochenour *et al.* 1962) the prophylactic regimen of penicillin employed was inadequate. Of the thirty-eight monkeys one remained well throughout and four experienced transient fever following cessation of the initial course of penicillin but recovered spontaneously. One monkey died from non-specific pneumonitis 3 days after cessation of penicillin. One animal died of anthrax septicaemia 48 hr. after exposure; this animal had received but a single dose of penicillin. Five other monkeys died 3–9 days following cessation of penicillin without having developed abnormal chest X-rays; death was due to anthrax septicaemia, meningitis, or both. These animals did not exhibit significant mediastinal or pulmonary involvement at autopsy, thus confirming clinical and radiographic impressions. One monkey died of anthrax involvement of the mediastinum 19 days after the initial course of penicillin; the last chest roentgenogram was obtained 4 days earlier and was normal.

Radiographic evidence of involvement of pulmonary and mediastinal structures was present in 25 (66 %) monkeys. The characteristic lesion of primary interest, mediastinitis, was manifested by a smooth, diffuse widening of the superior mediastinum (Pl. 1). Such a finding, with or without pulmonary lesions, was detected in nineteen animals. In six monkeys the only radiographic lesions were localized pneumonic infiltrates in the lower lobes (Pl. 2). Histopathologically these latter lesions correspond to the superimposition of anthrax bronchiolitis upon pre-existing lesions caused by the lung mite, *Pneumonyssus simicola* (Berdjis, Gleiser, Hartman, Kuehne & Gochenour, 1962). Since the process in such monkeys differs from that of woolsorter's disease, they are considered separately.

Mediastinitis was detected by X-ray as early as 3 and as late as 14 days (mean = 6 days) following cessation of the 'prophylactic' course of penicillin. Mediastinal

changes were first detected upon supervoltage films in fourteen (74%) of the nineteen animals which developed mediastinitis; in no case was the conventional film positive before the 1 MeV. film. The usefulness of the supervoltage technique was further emphasized in that the conventional films of twelve of these remained equivocal or negative. Of course, therapy (see below) undoubtedly halted the progression of lesions. In the case of monkeys with pulmonary parenchymal lesions the two film techniques appeared equally applicable.

Typically, fever preceded recognizable roentgen changes by a few hours to several days; three of the monkeys with chest lesions remained afebrile throughout and in eight animals chest lesions were detected prior to or coincident with the onset of fever. It is of practical importance that in only half the monkeys were blood cultures positive at the time chest lesions were first detected by X-ray. Otherwise there were no significant manifestations of illness in untreated monkeys until 12–24 hr. before death. At that time the apparently benign course of illness gave way to severe prostration and shock; tachypnea and cyanosis were common. The mean duration of life in untreated animals, after X-rays were positive, was 2 days (range = $\frac{1}{2}$–4$\frac{1}{2}$ days). The seeming diphasic course of this disease in monkeys, i.e. an apparently 'benign' febrile illness which suddenly turns into a fulminant terminal phase, parallels that of woolsorter's disease in man, further validating the experimental model.

At post-mortem examination untreated animals with clinical and radiographic signs of mediastinal involvement exhibited a gelatinous haemorrhagic mediastinitis (Pl. 3). There were, in addition, evidences of anthrax septicaemia. Pulmonary parenchymal lesions were variable accompaniments of mediastinitis. Those monkeys dying with primary pulmonary lesions exhibited the anthrax bronchiolitis previously mentioned in addition to evidences of septicaemia. In no case was significant mediastinitis detected in the absence of prior recognition of mediastinal involvement by chest X-rays.

Early antibiotic therapy. Since chest roentgenograms reveal lesions during the initial 'benign' phase of anthrax mediastinitis in monkeys and in man (Plotkin, Brackman, Utell, Bumford & Atchison, 1960) and such lesions are reasonably distinctive in appearance, they may serve as invaluable aids to early diagnosis. Accordingly X-ray detection of significant chest lesions was taken as the indication for institution of therapy in these studies. Penicillin, distinct from the earlier 5-day course of 'prophylaxis', was administered to monkeys with mediastinitis. Defervescence was prompt, within 48 hr., and chest films returned to normal in 1–7 days (mean = 3$\frac{1}{2}$ days). Two treated monkeys, however, ultimately died of anthrax. One expired with mediastinitis, septicaemia and meningitis 5 days after institution of penicillin therapy; of particular interest was the recovery at autopsy of *penicillin resistant B. anthracis* from this animal's spleen. The organisms recovered produce penicillinase and are resistant to penicillin in concentrations of 100 units per ml. The other death, due to meningitis, occurred 25 days after completion of an apparently successful course of therapy. Whether or not this death could have been prevented by administration of protective antigen at the time of illness is a matter of speculation; available evidence suggests it could

(Henderson, Peacock & Belton, 1956). In all, then, penicillin therapy instituted at the time of X-ray diagnosis of anthrax mediastinitis was successful in eight of ten monkeys. Such results differ from those in untreated animals wherein but 1 of 6 survived ($\chi^2 = 3{\cdot}81$;* $0{\cdot}06 > P > 0{\cdot}05$). The one untreated survivor had slight but definite mediastinal widening on 1 MeV. films over a 3-day period beginning the 12th post-exposure day; this monkey remained afebrile throughout.

Two of the three monkeys with mediastinitis treated with tetracycline survived. Defervescence and return of X-rays to normal appeared similar to that resulting from penicillin. The other died 4 days following initiation of therapy with tetracycline; post-mortem examination revealed haemorrhagic mediastinitis, lymphadenitis and evidence of septicaemia.

Of the six monkeys exhibiting only pulmonary lesions by X-ray, three were treated with tetracycline and survived. The three untreated animals died of pneumonia and septicaemia. X-rays of treated animals cleared in 2–7 days.

It is noteworthy that cultures of lung taken from *all* survivors of the study when they were killed, 55–84 days after exposure, were positive for *B. anthracis*. In none of these, however, was penicillin resistance detected. This evidence of prolonged spore retention, as well as that of others (Henderson *et al.* 1956), together with the late death of a 'successfully' treated monkey emphasizes the importance of an adequate antigenic experience in protection from inhalation anthrax as enunciated by Henderson and his co-workers, and by Gochenour *et al.* (1962).

DISCUSSION

Animal studies (Keppie, Smith & Harris-Smith, 1955) clearly indicate that to be successful antibiotic therapy of anthrax infections must be instituted during the initial phases of illness. Recent case reports of inhalation anthrax (Plotkin *et al.* 1960) suggest this to be true for man as well. Later in the illness antibiotic treatment, even though effective in reducing bacterial numbers, does not prevent death. The relatively non-specific initial symptoms of human inhalation anthrax renders early, and for that matter *ante mortem*, diagnosis difficult. As a result most patients have remained untreated until late in the illness; consequently death has been the rule.

The experimental model of woolsorter's disease in rhesus monkeys utilized here suggests that antibiotic therapy with either penicillin or tetracycline, begun at a time when distinct chest roentgen changes can be recognized, is efficacious. Such roentgen evidence appeared early in the course of illness and preceded the late fulminant course by 12–90 hr. Extrapolation from an animal model to human disease will always be limited to the extent to which the model parallels the disease in man. The model utilized herein simulated human inhalation anthrax to a degree unknown in other systems. Hence, the observation that chest roentogenographic changes permit recognition of the illness at a time when antibiotic therapy is effective seems particularly pertinent. Individuals potentially exposed to *B. anthracis* who experience respiratory symptoms deserve early and repeated, care-

* χ^2 test with correction for continuity.

fully obtained chest X-rays. An alertness to the possibility of woolsorter's disease and recognition of distinctive radiographic changes leading immediately to vigorous antibiotic treatment may make possible the control of this otherwise fatal illness.

Numbers of animals are inadequate to compare the efficacy of the two antibiotics, penicillin and tetracycline. One death occurred with each antibiotic several days after institution of therapy. In the case of the monkey dying while receiving penicillin, anthrax bacilli resistant to that antibiotic were recovered. This observation suggests that if penicillin were chosen as the therapeutic agent it should be supplemented with a second antibiotic; streptomycin, shown to be effective in the studies on guinea-pigs conducted by Keppie and his associates might be suitable. Alternatively, a broad spectrum antibiotic might be employed as the supplemental drug either initially or added subsequently if 'antagonism' is a concern. Since the mechanism of penicillin resistance of the organism encountered here was penicillinase production, the recently introduced penicillinase-resistant penicillins should be considered. A broad spectrum drug alone would appear efficacious though perhaps suboptimal, based on these limited studies.

Uniform presence of spores for many weeks in surviving animals and the late death emphasize the need for adequate antigenic stimulation in animals exposed to anthrax aerosols. In practice, all treated individuals should probably receive protective antigen after recovery in order to assure such stimulation.

The results of the present study suggest that supervoltage diagnostic film techniques are advantageous for the earliest detection of mediastinal soft tissue changes. Where appropriate equipment and personnel are at hand such techniques are to be recommended for suspected mediastinal infection. Such a recommendation does not imply, however, that conventional radiographic techniques do not detect the mediastinal lesions such as those of woolsorter's disease early enough for effective therapy.

SUMMARY

1. A model of anthrax infection in rhesus monkeys closely simulating human inhalation anthrax has been employed to study the course of the illness and methods of recognition and therapy.

2. Roentgenographic evidence of mediastinitis was detected early in the illness and served as a suitable indication for starting treatment.

3. Therapy with either penicillin or tetracycline appeared efficacious when started at the time chest roentgenograms first evidenced disease.

4. Isolation of penicillin-resistant *B. anthracis* from an animal dying during treatment with penicillin is reported.

REFERENCES

Berdjis, C. C., Gleiser, C. A., Hartman, H. A., Kuehne, R. W. & Gochenour, W. S., Jr. (1962). Pathogenesis of respiratory anthrax in *Macaca mulatta*. *Brit. J. exp. Path.* **43**, 515.

Greenfield, W. S. (1880). Further investigations on anthrax and allied diseases in man and animals. Lecture I. *Lancet*, ii, 965.

GREENFIELD, W. S. (1881). Further investigations on anthrax and allied diseases in man and animals. Lecture III. *Lancet*, i, 91.

GOCHENOUR, W. S., Jr., GLEISER, C. A. & TIGERTT, W. D. (1962). Observations on penicillin prophylaxis of experimental inhalation anthrax in the monkey. *J. Hyg., Camb.*, **60**, 29.

HENDERSON, D. W., PEACOCK, S. & BELTON, F. C. (1956). Observations on the prophylaxis of experimental pulmonary anthrax in the monkey. *J. Hyg., Camb.*, **54**, 28.

KEPPIE, J., SMITH, H. & HARRIS-SMITH, P. W. (1955). The chemical basis of the virulence of *Bacillus anthracis*. III. The role of the terminal bacteraemia in death of guinea-pigs from anthrax. *Brit. J. exp. Path.* **36**, 315.

PLOTKIN, S. A., BRACHMAN, P. S., UTELL, M., BUMFORD, F. H. & ATCHISON, M. M. (1960). An epidemic of inhalation anthrax, the first in the twentieth century. I. Clinical features. *Amer. J. Med.* **29**, 992.

EXPLANATION OF PLATES

PLATE 1

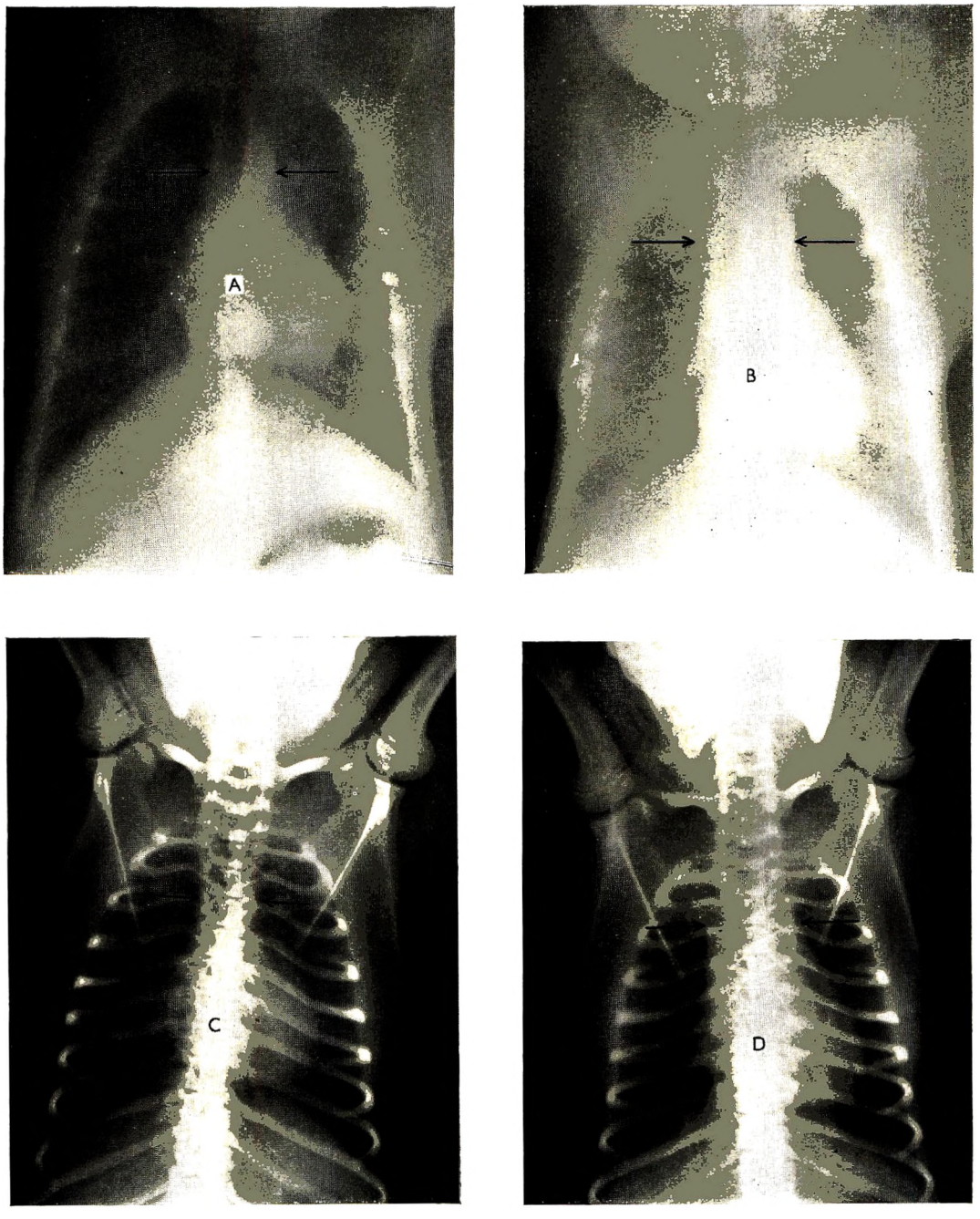
Radiographic appearance of Simian woolsorter's disease. A and C are normal. B and D reveal marked widening of the superior mediastinum. A and B are supervoltage films (1 MeV.); C and D are corresponding films obtained by conventional technique. Note the superior definition of soft tissues achieved with the supervoltage technique. (U.S. Army photograph.)

PLATE 2

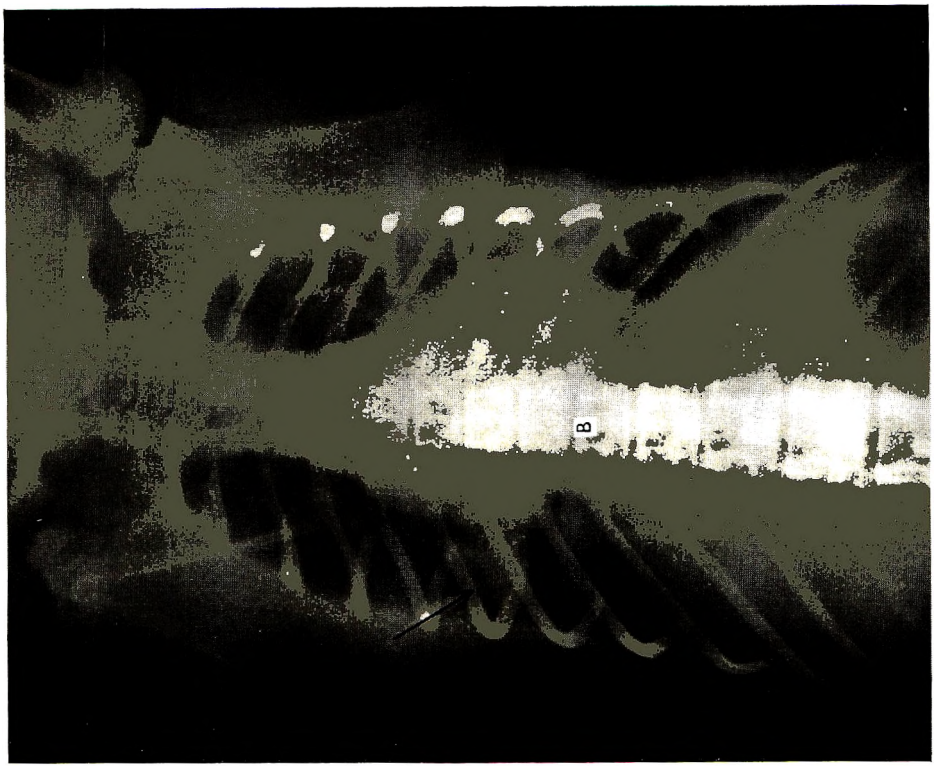
Pneumonic lesion due to *B. anthracis*. A is normal, B reveals an infiltrate adjacent to the right cardiac border. (U.S. Army photograph.)

PLATE 3

Post-mortem specimen of a monkey displaying marked mediastinitis. See Pl. 1 for radiographs of this animal. (U.S. Army photograph.)



(*Facing p.* 322)


B


A


Rec. 978
ALBER
USA Med. Unit
Ft. Detrick, Md.
& CO.
METALLURGICAL MICROSCOPES
PETROGRAPHIC MICROSCOPES
SLIDES AND COVER GLASSES
CLINICAL THERMOMETERS
MICROTOMES

J. Hyg., Camb. (1963), **61**, 323
Printed in Great Britain

An outbreak of human infection due to *Salmonella typhi-murium*, phage type 4, associated with the use of unpasteurized liquid egg

By A. J. ESSEX-CATER
Deputy Medical Officer of Health, City of Manchester

D. M. JONES
Public Health Laboratory Service, Manchester

F. SWINDELL
Senior Food Inspector, City of Manchester

(*Received* 8 *March* 1963)

INTRODUCTION

Salmonella typhi-murium is the most common salmonella serotype associated with food poisoning in England and Wales (Reports, 1961, 1962). The major part of the disease due to this organism is sporadic in distribution. In 1961, for example, out of 2503 incidents due to *S. typhi-murium*, 2463 were either sporadic cases or family outbreaks.

Of twenty fatal cases of food poisoning reported in 1961, seven were due to *S. typhi-murium*.

Eggs and egg products, poultry and meat, are known to be often infected with *S. typhi-murium* and bakeries may become contaminated from such sources (Philbrook *et al.* 1960; Harvey & Philips, 1961). As Anderson (1962) has pointed out, tracing the source of *S. typhi-murium* infection is made difficult by the widespread reservoir of infection in livestock and also because food is often prepared in an environment which has been contaminated by infected raw materials. A single bakery may distribute its products widely so that if they are contaminated the epidemiological picture is one of multiple sporadic infections and the unified nature of the outbreak may be obscured.

The value of phage-typing in tracing the source of *S. typhi-murium* was demonstrated in an outbreak due to infected meat described by Anderson, Galbraith & Taylor (1961). In the outbreak of *S. typhi-murium* infection described here the source would have remained undetected without the aid of phage-typing. The cases were scattered throughout the south-east Lancashire conurbation and were associated with unpasteurized English liquid egg from one producer.

SALMONELLA INFECTION IN MANCHESTER AREA 1962

The pattern of infection in the conurbation in 1962 was similar to that elsewhere in the United Kingdom; most of the infection was apparently sporadic. Epidemiological and bacteriological investigations conducted during the year finally revealed that much of this sporadic infection was related to, and emanated from, a single source of unpasteurized liquid English egg.

Seventy-two incidents due to *S. typhi-murium* occurred during the year and twenty-seven of these were found to be due to phage type 4. Incidents due to this type comprised thirty-two cases and twelve symptomless excreters in the conurbation. More than half the clinical cases were in children but only one of the symptomless excreters was a child. No single incident accounted for more than three cases, although one family outbreak included two cases and two symptomless excreters and five symptomless excreters were employees of one infected bakery. The monthly incidence of food poisoning in the south-east Lancashire conurbation in 1962 due to *S. typhi-murium* (all phage types) is illustrated in Fig. 1.

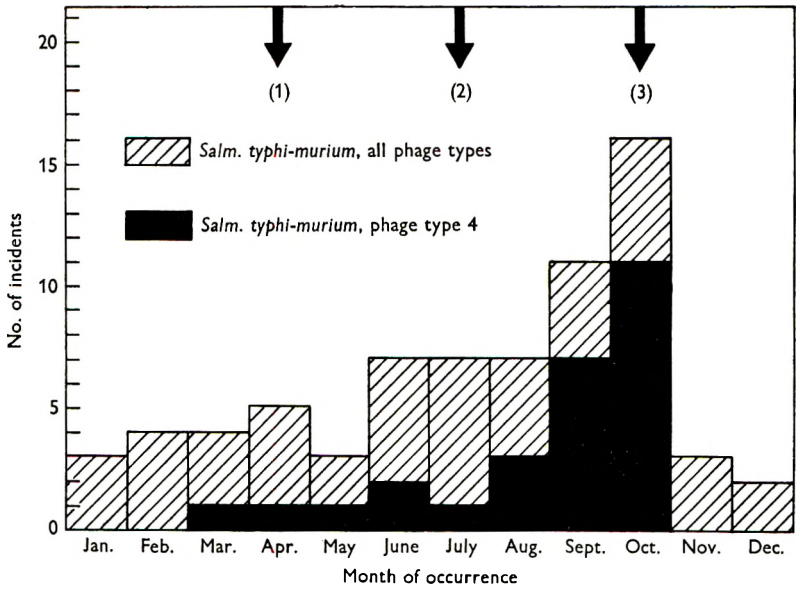


Fig. 1. *S. typhi-murium* food poisoning incidence, south-east Lancashire conurbation, 1962. (1) Producer supplying one bakery. (2) Producer supplying eight bakeries. (3) Producer's liquid egg pasteurized and bakeries disinfected.

Twelve incidents due to phage type 4 embracing fourteen clinical cases and eleven symptomless excreters occurring in the city of Manchester are detailed in Table 1 and were subjected to special study.

NOTES ON CLINICAL CASES

In case 1, a Sonne dysentery infection was also reported by the laboratory in addition to *S. typhi-murium*. Case 2 was seriously ill and required intravenous therapy in hospital, while case 4 presented with a scarlatiniform rash. In two cases (5 and 12) a long period of diarrhoea and associated abdominal symptoms preceded the detection of the organism.

Table 1. *City of Manchester. Food poisoning incidents due to* Salmonella typhi-murium, *phage type* 4, 1962

Incident number	Clinical case or symptomless excreter number	Date of onset or laboratory diagnosis	Age	Sex	Source of infecting agent or other relevant information
I	Case 1	4. viii. 62	2 years	M	Unknown
II	Case 2	17. viii. 62	Adult	M	Had meals at establishments, some of which obtained foods from suspect bakeries
III	Case 3	22. viii. 62	Adult	F	Confectionery from bakery using dried egg from infected liquid egg producer
IV	Case 4	6. ix. 62	2 years	F	Unknown
	S.E. 1	17. ix. 62	9 months	F	Sister of case 4
V	Case 5	18. ix. 62	20 months	M	Confectionery from suspect bakery
VI	Case 6	19. ix. 62	5 years	M	Confectionery from suspect bakery
	Case 7	25. ix. 62	6 years	F	Sister of case 6
	S.E. 2	8. ix. 62	Adult	F	Mother of cases 6 and 7
	S.E. 3	8. ix. 62	Adult	F	Nurse of cases 6 and 7
VII	Case 8	28. ix. 62	19 years	F	Ate infected trifle
	Case 9	5. x. 62	Adult	F	Room-mate of case 8
	S.E. 4	11–15. x. 62	Adult	M	Operative, suspect bakery (A)
	S.E. 5			F	
	S.E. 6			M	
	S.E. 7			M	
	S.E. 8			M	
	S.E. 9	12. x. 62		F	Resided with symptomless excreter 4
	S.E. 10	22. x. 62		M	Operative, suspect bakery (B)
VIII	Case 10	1. x. 62	11 years	M	Confectionery from suspect bakery (A)
	S.E. 11	17. x. 62	Adult	F	Grandmother case 10
IX	Case 11	9. x. 62	Adult	M	Had meal at cafe supplied by suspect bakery
X	Case 12	Early x. 62	11 years	M	Confectionery from suspect bakery
XI	Case 13	Mid. x. 62	2 years	M	Confectionery (?) from suspect bakery
XII	Case 14	28. x. 62	Adult	M	Uncertain

ACCOUNT OF THE INVESTIGATION

A number of scattered and apparently unrelated incidents due to *S. typhi-murium*, type 4, had occurred over several months in the conurbation, but no linking factors between them had been found and the source of infection was unknown.

A young resident (case 8) at a hostel for unmarried mothers, administered by the city, became suddenly ill with pyrexia and diarrhoea two days after dining at a café in the city. Apart from this she had taken all meals with other residents at the hostel, none of whom was indisposed. *S. typhi-murium*, type 4, was isolated from her faeces. A trifle sampled at the café also contained this organism. As a result, the bakery which prepared the trifle was investigated, and *S. typhi-murium* was found in samples of liquid egg and in swabs taken from mixing machines, bowls, whisks and table surfaces. Five symptomless excreters were found amongst twenty-one employees in the bakery. The infected unpasteurized liquid egg used in the bakery, almost certainly the immediate agent of infection, was issued by a firm in the area of a neighbouring authority. Inspectors from this authority found that the producer was distributing unpasteurized liquid egg to a steadily widening field of bakeries in the area. The eggs used were rejects obtained from an egg-collecting station in Yorkshire. These were mostly hen's eggs but also included some duck and turkey eggs. The realization that infection was probably widely scattered through many bakeries in the area called for an investigation of such premises.

All 224 bakehouses in the city were visited to discover what type of egg products were used. Ninety-one of these bakeries used liquid egg, twenty-two dried egg and sixteen fresh egg; the remaining ninety-three did not use egg.

In view of the large number of bakeries concerned, preliminary inquiries were made at the larger establishments first, samples of liquid and dried egg being examined and members of the staff being required to submit faecal specimens. Infection was detected in samples from three moderate-sized bakeries. In one of the bakeries a further symptomless excreter of *S. typhi-murium*, type 4, was detected among seven operatives and he was excluded from work pending the examination of three successive negative faecal specimens over a period of 8 days.

Although conditions at the bakehouses visited were generally satisfactory, it was evident that, apart from six large bakeries which mainly used pasteurized egg, few proprietors appreciated the potentialities of unpasteurized liquid egg as a cause of food poisoning. They were advised to insist on being supplied in future with pasteurized egg and to take adequate steps to sterilize any equipment liable to contamination.

In order to assess the bacteriological condition of the liquid or frozen bulked egg mainly used at the other smaller bakeries, it was considered more practical to examine samples obtained at the focal points of supply within the city rather than at the bakeries themselves. As this outbreak resulted from home-produced egg, attention was directed to this in the first instance, imported egg being subject to some control at the port of entry.

It was found that an egg producers' marketing organization had stocks of English and imported egg in a city cold store at the disposal of five dealers who supplied fifty-four of the ninety-one bakeries. From the middle of October to the end of December 1962, samples were examined bacteriologically from 272 tins representing a 5% selection of 68 tons (40 batches) of English frozen egg at the cold store. *S. typhi-murium*, phage types 1 var. 5 and 12a, was found in nineteen samples; 7 tons (3 batches) of the egg were therefore detained pending satisfactory arrangements for pasteurization.

This programme of sampling home-produced liquid egg and associated products will be continued until pasteurization becomes a routine measure.

Laboratory investigations

Swabs taken from the bakery utensils and tables were cultured overnight at 37° C. in selenite F medium. Subcultures were made on to deoxycholate-citrate agar and Wilson and Blair medium and these were then incubated overnight. From each sample of liquid egg 10 ml. were distributed into each of three universal containers. To two of these, 10 ml. double-strength selenite medium were added: one was incubated at 37° C. and the other at 43° C. To the third universal container 10 ml. tetrathionate medium were added followed by incubation at 37° C. Subcultures were made next day on to deoxycholate-citrate agar and Wilson and Blair medium. Colonies suspected of being salmonellae were examined biochemically and serologically. Cultures of *S. typhi-murium* were sent to the Enteric Reference Laboratory, Colindale, for phage-typing.

Pasteurization of liquid egg

Eggs are broken out, the shell is smelt for soundness and the liquid egg passed or rejected accordingly.

The liquid egg is then bulked and emulsified before heating in a plate exchange apparatus to 60–65° C. The process is thermostatically controlled and the liquid is held at this temperature for 4 min. and then cooled to about 6° C., unless the egg is to be spray-dried, when it is not cooled below 16° C.

After pasteurization the liquid egg is run into sterilized tins which are sealed and refrigerated.

Sampling should be undertaken by the local health authority and the product not released for sale until purity is confirmed.

DISCUSSION

The outbreak described here indicates once more that liquid egg is an important source of human infection. This danger has been long recognized (Report, 1955, 1958, 1959). The point that emerges is that much of the illness from this source is sporadic in distribution and its epidemiological significance is easily missed. Anderson (personal communication) states that phage type 4 is commoner in ducks than in hens, and, as it is likely that duck eggs were included in the unpasteurized

eggs responsible for the type 4 infections, it is possible that they were responsible for the introduction of the infecting organism. The importance of looking for environmental contamination where food is produced cannot be over-emphasized.

The producer of this infected liquid egg started in business in April 1962, and at first supplied one bakery. He drew his eggs for breaking from the same source as a pasteurizing plant near Manchester. Liquid egg sampled before pasteurization at this plant in April was shown to be contaminated with *S. typhi-murium*, phage type 4. Thus, by inference, the egg distributed by the producer was contaminated with this organism from the onset. By July he was supplying eight bakeries. From the diagram (Fig. 1) it can be seen that about this time the number of incidents due to type 4 began to increase. When investigated, in October, only three bakeries showed evidence of environmental contamination. These three bakeries used more egg than the others and in each of them some of the staff were found to be excreting the organism. Two of these bakeries were associated with cases of food poisoning in the population. The third bakery produced only bread, using the contaminated egg as a glaze. In this bakery there were no opportunities for cross-infection of other products. When the source of the contaminated egg became known, the producer immediately submitted his product for pasteurization. At this time also, the contaminated bakeries were cleaned and utensils sterilized. After this, no more cases of type 4 infection were notified.

A retrospective survey of earlier incidents of *S. typhi-murium*, type 4, food poisoning was made in Manchester. Of fourteen cases nine were certainly infected via contaminated bakeries and three others probably were. The association was not absolute for two reasons. First, in some instances there was a lapse of 2 months or more before the relevant inquiry was made and, secondly, the producer in question was probably not the only source of type 4 infection in Manchester, as this type had been isolated in previous years.

Unlike the salmonella infection of meat and poultry, the contamination of egg products can be controlled effectively by pasteurization. Efficient methods for this have been developed and standardized (Heller, *et al.* 1962) and plant is becoming available to carry them out. Bacteriological and chemical tests make the control of the product a simple matter.

The pasteurized product is not inferior in baking quality but the opposition to its use amongst bakers questioned in this inquiry appeared to be based on conservatism and ignorance. In fact, many of the bakers had at times, unknown to themselves, been supplied with pasteurized egg which they used without comment. Although the Manchester investigation revealed an imperfect standard of hygiene in certain bakeries, the complete adoption of adequate hygienic measures in bakeries would not alone be sufficient to ensure the safety of the community from food poisoning through the use of unpasteurized egg products. There is little doubt that the use of liquid egg is increasing and it appears essential that no liquid egg should be issued for use in the confectionery or related trades before it has been effectively pasteurized. There are sufficient plants in the country capable of pasteurizing liquid egg and it is hoped that prompt legislation will be introduced so that one more gap in food hygiene is eliminated.

Under the Public Health (Imported Food) Regulations, it is an offence to import any article of food for sale for human consumption which has been examined and not found to be fit. Ross (1962) records that since 1955, 75 salmonella serotypes have been isolated from imported egg products; only 20 of these serotypes have not been associated with human infection. Imported eggs and egg products are covered by these Regulations.

No existing legislation requires the pasteurization or other suitable treatment of home produced eggs and egg products, and only the broad protective measures of the Food and Drugs Act, 1955, exist for dealing with them.

The present investigation taken together with the results of other inquiries, indicates that home-produced liquid egg is often infected with salmonella organisms and emphasizes the need for severe restrictions on the commercial use of unpasteurized liquid egg and associated products. Certainly duck and turkey eggs, more likely to be infected with pathogens, should be excluded from liquid egg. According to Masár (personal communication) duck eggs cannot be used in food intended for human consumption in Czechoslovakia or Russia.

SUMMARY

During 1962, twenty-seven food poisoning incidents due to *Salmonella typhi-murium*, phage type 4, comprising thirty-two cases and twelve symptomless excreters, occurred in the south-east Lancashire conurbation. The source of infection was a single small producer of English liquid egg. Pasteurization of the product coincided with the end of the outbreak. The cases were sporadic in distribution. Investigation of previous incidents due to this phage type in the City of Manchester pointed to egg from the same producer. As a result an investigation was made into the amount and source of unpasteurized egg in use in the city. This revealed the frequent presence of salmonella infection and demonstrated the need for pasteurization of liquid egg before use.

We are indebted to Dr E. S. Anderson and the staff of the Enteric Reference Laboratory, Colindale, for phage-typing the strains of *S. typhi-murium*, so essential to this investigation: also to the food inspectors of the City of Manchester and to Mr Hobson, Chief Public Health Inspector, Salford.

REFERENCES

Anderson, E. S. (1962). The use of bacteriophage typing in the investigation of outbreaks. In *Food poisoning*, Royal Society of Health, London.

Anderson, E. S., Galbraith, N. S. & Taylor, C. E. D. (1961). An outbreak of human infection due to *Salmonella typhi-murium* phage-type 20*a*, associated with infection in calves. *Lancet*, i, 854.

Harvey, R. W. S. & Philips, W. P. (1961). An environmental survey of bakehouses and abattoirs for salmonellae. *J. Hyg., Camb.*, **59**, 93.

Heller, C. L., Roberts, B. C., Amos, A. J., Smith, Muriel A. & Hobbs, Betty C. (1962). The pasteurization of liquid whole egg and the evaluation of the baking properties of frozen whole egg. *J. Hyg., Camb.*, **60**, 135.

PHILBROOK, F. R., MACCREADY, R. A., VAN ROEKEL, H., ANDERSON, E. S., SMYSER Jr., C. F., SANEN, F. J. & CROTON, W. M. (1960). Salmonellosis spread by a dietary supplement of avian source. *New Engl. J. Med.* **263**, 713.

REPORT (1955). Salmonella in liquid egg, including frozen liquid egg. *Mon. Bull. Minist. Hlth Lab. Serv.* **14**, 65.

REPORT (1958). The contamination of egg products with salmonellae, with particular reference to *Salm. paratyphi B. Mon. Bull. Minist. Hlth Lab. Serv.* **17**, 36.

REPORT (1959). The examination of English egg products. *Mon. Bull. Minist. Hlth Lab. Serv.* **18**, 158.

REPORT, (1961). Food poisoning in England and Wales, 1960. *Mon. Bull. Minist. Hlth Lab. Serv.* **20**, 160.

REPORT (1962). Food poisoning in England and Wales, 1961. *Mon. Bull. Minist. Hlth Lab. Serv.* **21**, 180.

ROSS, J. M. (1962). Some problems in the bacteriological sampling of imported foods. *Report, A.G.M., Ass. Sea, Air Port Health Authorities, British Isles.*

J. Hyg., Camb. (1963), **61**, 331
Printed in Great Britain

Infectivity of influenza virus aerosols

By A. M. HOOD
Microbiological Research Establishment, Porton Down, Salisbury, Wilts

(*Received* 14 *March* 1963)

The PR8 strain of influenza virus can remain viable in ageing aerosols for considerable periods (Harper, 1961). However, viability of micro-organisms determined by their growth *in vitro* may not indicate their ability to infect animals via the respiratory route (Schlamm, 1960; Hood, 1961). If influenza is normally transmitted via this route it is epidemiologically important to establish whether any correlation exists between viability as measured by *in vitro* or *in ovo* methods and respiratory infectivity for a susceptible animal host. Previous reports on respiratory infectivity of aged influenza virus aerosols by Edward, Elford & Laidlaw (1943), Loosli, Lemon, Robertson & Appel (1943*a*) and Loosli, Robertson & Puck (1943*b*) lack such a comparison.

Recently developed *in vitro* techniques for influenza virus assay (Fazekas de St Groth & White, 1958*a*), for holding aerosols for long periods (Goldberg, Watkins, Boerke & Chatigny, 1958) and for assessing physical decay of aerosols (Harper, Hood & Morton, 1958) facilitate a study of this kind. This paper describes an attempt to determine whether two strains of influenza virus lose respiratory infectivity with ageing in aerosols.

MATERIALS AND METHODS

Two mouse-passaged strains of influenza virus, PR8 (type A) and Asian (Singapore, type A2), were used. Preparation of viral suspensions *in ovo*, production, holding, sampling and physical decay measurement of aerosols were similar to those described by Harper (1961) except that dialysed casein was replaced by 0·2% (w/v) gelatin in the suspending and collecting fluids and a stainless steel drum of 500 l. capacity was used for holding the aerosols. Several batches of suspension were used, storage time (in solid CO_2) before use varied from 9 to 62 days for the PR8 strain and 1 to 40 days for the Asian strain. Virus titres of suspensions used for generating aerosols and of aerosol samples were determined *in vitro* using the egg-membrane piece technique (Fazekas de St Groth & White, 1958*a*). Membrane pieces (MP) from 13-day-old eggs were used for the PR8 strain and from 12-day-old eggs for the Asian strain. Frequent parallel assays were made by allantoic injection *in ovo*. The titres of the virus suspensions used were from 100 to 400×10^6 EIU50/ml., 7 to 50×10^6 MP50/ml. for the PR8 strain and 40 to 200×10^6 EIU50/ml., 3 to 50×10^6 MP50/ml. for the Asian strain.

Aerosols were held at approximately 23° C. under dry (20% R.H.), medium (50% R.H.) and wet (80% R.H.) conditions. Respiratory infectivity (LD50 and ID50) of the aerosols was obtained by exposing 18–22 g. mice, in groups of 8–10 for

periods ranging between 10 sec. and 20 min., in a manner similar to that previously described (Hood, 1961) for exposing guinea-pigs. After exposure the mice were held for 3–4 weeks in individual isolation boxes and deaths recorded. The lung-retained minute volume for mice of this weight inhaling particles of the size generated in the apparatus was shown to be *ca.* 7 ml. (Harper & Hood, 1962). The dose of virus particles retained by the mouse was obtained by the product of: 7 × exposure time (min.) × viable virus/ml. of aerosol.

In some experiments mice surviving 3–4 weeks after exposure to small doses of virus were bled and sera examined for the presence of haemagglutination-inhibiting influenza virus antibodies against the homologous strain of virus. Since negative control sera were < 5 in titre, antibody titres of > 20 were accepted as evidence of infection.

RESULTS

PR 8 *strain of influenza virus*

Determination of viable virus by the MP method resulted in aerosol viabilities similar to those reported by Harper (1961) (Table 1). Virus titre ratios—*in ovo*/MP—for suspensions and aerosol samples did not differ significantly and were

Table 1. *Mouse respiratory LD*50 *of PR*8 *influenza virus aerosols*

Suspension*	Aerosol conditions: Temp. (°C.)	R.H. (%)	Viability (%)	Age (hr.)	LD50 (in MP50 units)	Relative potency whole egg/egg membrane piece of aerosol sample
A	23·3–25	15–21	40	0	4 (0·7–10)	3 (1–8)
A	—	—	8	20	3·5 (1·1–12)	6 (3–12)
A	—	—	160	0	1·2 (0·5–3)	7 (3–15)
A	—	—	12	20	7·8 (3·3–18)	5 (2–10)
B	—	—	74	0	2·9 (1·4–10)	4 (1–9)
B	—	—	11	20	2·2 (0·9–4·4)	8 (3–22)
B	—	—	13	20	2·1 (0·3–6·7)	—
B	—	—	44	0	1·6	—
B	—	—	12	20	1·4 (0·5–3)	—
C	22–25	52–55	30	0	2·8 (1·3–6·5)	8 (4–15)
C	—	—	4	1	4·4 (1·8–15·6)	—
D	—	—	77	0	7·3 (2·3–59)	2 (1–4)
D	—	—	1	2	3·5 (1·1–4·8)	8 (4–17)
C	23·3–26·6	78–85	52	0	4·8 (1·9–38)	6
C	—	—	2	4	2·2 (0·8–14)	—
C	—	—	48	0	2·4 (1–6)	—
C	—	—	1	4	1·1 (0·5–3·5)	8 (4–17)
C	—	—	44	0	1·7 (0·4–∝)	8 (4–15)
C	—	—	0·1	4	2 (0·2–∝)	—
E	—	—	35	0	4·7 (1–3917)	20 (8–53)
E	—	—	4	2	2·8 (1–∝)	—

Weighted mean LD50 = 2·6 (2–3) MP 50 units.
0 hr. = *ca.* 3 sec.
Figures in parentheses are 95% confidence limits.
* Relative potency (whole egg/egg membrane piece) of 10 suspension samples = 11·8 (range 2·4–31).

similar in range to those reported by Finter & Armitage (1957), and Fazekas de St Groth & White (1958*b*) for this virus. Thus, no change in the relative sensitivity of the ageing aerosol virus to the two methods of assay was found.

The mouse respiratory LD50 for aerosols aged 3 sec. was not significantly different from those at 2 hr. (50% R.H.), 4 hr. (80% R.H.) and 20 hr. (20% R.H.). All were within the range of 1–8 MP50 units. The weighted mean of the LD50's for all aerosol ages was 2·6 MP50's with 95% confidence limits of 2–3·4. Mouse deaths occurred between the sixth and fifteenth day after exposure.

The presence of haemagglutination-inhibiting influenza antibodies in blood sera of surviving mice indicated that the ID50 was about 0·03–0·04 MP50 for all aerosol test conditions, i.e. about 1–2% of the LD50 (Table 2).

Table 2. *Respiratory infectivity (mouse) of PR8 influenza virus*

Aerosol conditions			
R.H. (%)	Age (hr.)	Mouse dose (MP50)	Mice infected (%)
18	20	0·08	90
49	0	0·02	100
50	0	0·04	40
55	1	0·08	90
85	0	0·04	90
85	0	0·05	90
85	2	0·04	60
85	4	0·1	60
85	4	0·01	0

0 hr. = *ca.* 3 sec.

The Asian strain of influenza

The virulence (lethality) of the Asian strain was less than that of the PR8 strain and suspension titres several-fold lower. To obtain aged aerosols containing sufficient viable virus to enable mouse respiratory LD50's to be determined with reasonable exposure times more concentrated (25 and 50% allantoic fluid) suspensions had therefore to be used. These suspensions were unstable and had relatively short storage lives. It was thus rarely possible to repeat tests with any one batch. The LD50's obtained ranged from 3 to 75 MP50's but this variation was not associated with age or R.H. of the aerosols (Table 3). Statistical assessment showed the data to be heterogeneous (unlike those obtained with PR8) and the relevant heterogeneity factor had to be used when calculating the 95% confidence limits of the weighted mean LD50. These limits were 6·4–17 and the weighted mean was 10 MP50's. A few tests of surviving mice for blood sera antibody titre indicated ID50's of *ca.* 0·5 MP50, i.e. about 5% of the LD50 for all types of aerosol (Table 4). The *in ovo*/MP titre ratios confirmed that previously found with the PR8 strain, i.e. no change in the relative sensitivity of ageing virus from aerosols to the two assay methods. Aerosol viability of the Asian strain was similar to the PR8 strain under similar test conditions.

Table 3. *Mouse respiratory LD50 of Asian influenza virus aerosols*

Suspension*	Aerosol conditions: Temp. (°C.)	R.H. (%)	Viability (%)	Age (hr.)	LD50 (in MP50 units)	Relative potency whole egg/egg membrane piece, of aerosol sample
1	21–23	13–26	45	0	8 (4–20)	13 (6–32)
2	—	—	20	20	41 (21–493)	9 (4–22)
3	—	—	71	0	41 (14–169)	8 (5–12)
3	—	—	12	20	7 (2–82)	24 (10–72)
4	—	—	100	0	26 (9–143)	—
5	—	—	61	0	75 (32–358)	—
5	—	—	21	20	6 (2–16)	13 (8–21)
4	21·7–24	49–51	27	0	9 (0·4–1807)	15 (8–31)
4	—	—	8	1	6 (2–67)	9 (5–18)
5	—	—	37	0	45 (22–409)	—
5	—	—	3	1¼	6 (4–12)	—
6	—	—	39	0	3 (1–16)	—
6	—	—	13	1	11 (3–347)	—
3	20·6–25	82–88	29	0	17 (5–326)	22 (12–43)
3	—	—	7	4	8 (2–99)	2 (0·3–7·8)
3	—	—	3	4	3 (1–18)	8 (3–25)
4	—	—	73	0	21 (11–55)	—
5	—	—	103	0	33 (9–156)	—
5	—	—	13	¾	15 (5–162)	17 (10–29)

Weighted mean LD50 = 10 (6–17, with the relevant heterogeneity factor), MP 50 units.
0 hr. = *ca.* 3 sec.
Figures in parentheses are 95 % confidence limits.
* Relative potency (whole egg/egg membrane piece) of 10 suspension samples = 14·8 (range 4·3–30).

Table 4. *Respiratory infectivity (mouse) of the Asian strain of influenza*

Aerosol conditions: R.H. (%)	Age (hr.)	Dose MP50's	% mice infected	ID50
21	0	1·9	80	—
21	20	0·6	44	—
26	0	—	—	1·6 (0·6–4·7)
26	20	—	—	0·3 (0·2–0·5)
57	0	0·5	60	—
50	2	0·8	90	—
80	0	0·4	55	—
80	4	0·2	80	—
85	2	—	—	0·2

0 hr. = *ca.* 3 sec.

CONCLUSIONS

The ability of ageing aerosols of two strains (PR8 and Asian) of influenza virus to infect mice via the respiratory route is paralleled by their ability to grow *in vitro* (MP) and *in ovo*. The mouse respiratory LD50 of the viruses does not change significantly during ageing of aerosols up to 20 hr. under the conditions tested. There is also no apparent change in the ID50. Thus, the changes in viability of the viral aerosols (determined by MP and *in ovo* methods) were a direct indication of the respiratory infectivity and virulence of the aerosols for mice.

My thanks are due to Mr C. D. Kimber and Mr R. F. W. Southey for technical assistance and to Mr. S. Peto for statistical analysis.

REFERENCES

EDWARD, D. G. ff., ELFORD, W. J. & LAIDLAW, P. P. (1943). Studies on air-borne virus infections. 1. Experimental technique and preliminary observations on influenza and infectious ectromelia. *J. Hyg., Camb.*, **43** 1.

FAZEKAS DE ST GROTH, S. & WHITE, D. O. (1958*a*). An improved assay for the infectivity of influenza viruses. *J. Hyg., Camb.*, **56**, 151.

FAZEKAS DE ST GROTH, S. & WHITE, D. O. (1958*b*). Comparison of the infectivity of influenza viruses in two host systems: the allantois of intact eggs and surviving allantois-on-shell. *J. Hyg., Camb.*, **56**, 535.

FINTER, N. B. & ARMITAGE, P. (1957). The membrane piece technique for *in vitro* infectivity titrations of influenza virus. *J. Hyg., Camb.*, **55**, 434.

GOLDBERG, L. J., WATKINS, H. M. S., BOERKE, E. E. & CHATIGNY, M. A. (1958). The use of a rotating drum for the study of aerosols over extended periods of time. *Amer. J. Hyg.* **68**, 85.

HARPER, G. J. (1961). Airborne micro-organisms: Survival tests with four viruses. *J. Hyg., Camb.*, **59**, 479.

HARPER, G. J. & HOOD, A. M. (1962). Lung retention in mice exposed to airborne micro-organisms. *Nature, Lond.*, **196**, 598.

HARPER, G. J., HOOD, A. M. & MORTON, J. D. (1958). Airborne micro-organisms: A technique for studying their survival. *J. Hyg., Camb.*, **56**, 364.

HOOD, A. M. (1961). Infectivity of *Pasteurella tularensis* clouds. *J. Hyg., Camb.*, **59**, 497.

LOOSLI, C. G., LEMON, H. M., ROBERTSON, O. H. & APPEL, E. (1943*a*). Experimental airborne influenza infection. 1. Influence of humidity on survival of virus in air. *Proc. Soc. exp. Biol., N.Y.*, **53**, 205.

LOOSLI, C. G., ROBERTSON, O. H. & PUCK, T. T. (1943*b*). The production of experimental influenza in mice by inhalation of atmospheres containing influenza virus dispersed as fine droplets. *J. infect. Dis.* **72**, 142.

SCHLAMM, N. A. (1960). Detection of viability in aged or injured *Pasteurella tularensis*. *J. Bact.* **80**, 818.

J. Hyg., Camb. (1963), **61**, 337
Printed in Great Britain

The use of acetylethyleneimine in the production of inactivated foot-and-mouth disease vaccines

By F. BROWN, N. St G. HYSLOP, JOAN CRICK and A. W. MORROW
Research Institute (Animal Virus Diseases), Pirbright, Surrey

(*Received* 8 *March* 1963)

INTRODUCTION

The virucidal properties of formalin are widely employed in the production of inactivated vaccines against numerous virus diseases, although the uncertainty which accompanies its use for the *complete* inactivation of viruses is well known. This uncertainty was tragically emphasized by one of the earlier vaccines prepared against poliomyelitis. Since Vallée, Carré & Rinjard (1926) demonstrated that the virus of foot-and-mouth disease (FMD) could be inactivated with dilute formalin to give a product which would immunize cattle, this agent has been used extensively for the production of inactivated vaccines against the disease. In most laboratories concerned with the production of FMD vaccines the virus is adsorbed on to aluminium hydroxide gel before incubation with dilute formalin for periods up to 72 hr. (Waldmann & Köbe, 1938). Doubts have been expressed about this procedure because of the possibility that traces of residual virus might remain undetected owing to its combination with the adjuvant, and there is evidence that infective virus is present in vaccines prepared in this way (e.g. Moosbrugger, 1948; Schneider, 1955). Wesslen & Dinter (1957) showed clearly that complete inactivation of FMD virus is likely to be a prolonged process and some of our early inactivation experiments, carried out in the absence of aluminium hydroxide, showed that infective virus could be detected (by intradermal inoculation of cattle) after prolonged periods of incubation with 0·05% formalin. It seemed important, therefore, to examine alternative methods of inactivation which would be more certain to yield regularly a non-infective product.

Preliminary experiments showed that one strain of FMD virus could be inactivated extremely rapidly with 0·05% acetylethyleneimine (AEI) at 37° C. with the production of an effective vaccine for guinea-pigs (Brown & Crick, 1959). These findings have now been extended to other strains of the virus, grown in tissue culture, with the aim of producing vaccines for cattle.

MATERIALS AND METHODS

Virus production

Virus of strains 997 (type C) and Pak 1 (type Asia 1) was grown in cultivated pig kidney cells (Sellers, 1955) or in surviving bovine tongue epithelium fragments (Frenkel, 1951). The pig kidney cells were grown as monolayer cultures in Roux

flasks and inoculated with virus after 6–8 days. Virus was harvested when the monolayers were completely destroyed and the cell debris then removed by centrifugation. The production of virus in surviving tongue epithelium cells has been described in detail by Frenkel and his colleagues. In the present experiments, virus was harvested 18 hr. after infection.

Virus titration

Infectivity titrations were carried out by the intraperitoneal inoculation of groups of 7-day-old mice with tenfold serial dilutions of the virus (Skinner, 1951).

Virus inactivation

(*a*) Inactivation with AEI was carried out with 0·05 % (v/v) solutions at pH 7·6 and 37° C. and the reaction was stopped after the appropriate interval by the addition of $\frac{1}{10}$th volume of 20 % (w/v) sodium thiosulphate.

(*b*) For inactivation with formalin, virus suspensions containing 0·05 % (v/v) formalin were incubated at pH 7·6 or pH 9 for periods varying from 48 to 144 hr. at 26° C. The formalin was then neutralized with excess sodium bisulphite. For the preparation of vaccines similar to those available commercially, an equal volume of aluminium hydroxide gel was added to the virus suspension before incubating with 0·05 % (v/v) formalin at 26° C.

Innocuity tests

All the vaccines used in the guinea-pig experiments were tested for innocuity by intraperitoneal inoculation of 7-day-old mice (10–50 animals for each vaccine) and by intradermal inoculation of guinea-pig pads. In the cattle experiments innocuity tests were made by inoculating 0·1 ml. samples at twenty separate sites on the tongues of each of six steers. The significance of these tests has been discussed by Henderson (1952).

Potency tests

Groups of guinea-pigs or steers were inoculated subcutaneously with dilutions of vaccine. Those vaccines which were tested for innocuity in the absence of aluminium hydroxide were mixed with this adjuvant before inoculation. Blood samples were taken at 21 days and the animals were then challenged by intradermal inoculation of one hind pad (guinea-pigs) or of ten sites on the tongue (cattle) with 10,000 ID 50 of virus. The animals were then examined daily for the appearance of lesions typical of foot-and-mouth disease. In one experiment the cattle were not challenged but the virus neutralizing antibody levels of their sera were determined at intervals.

Serum neutralization tests

Specific neutralizing antibody was estimated by two methods:

(*a*) Dilutions of the homologous virus were mixed with an equal volume of 1/10 serum and the mixtures inoculated intraperitoneally into mice. The depression in virus titre produced by the serum was taken as the neutralization index of 1/20 serum (Skinner, 1953).

(*b*) In the second method, twofold dilutions of serum were mixed with 100 ID 50 of the homologous virus and the mixtures tested for residual virus in suspensions of cultivated pig kidney cells (Martin & Chapman, 1961). The end-point was the highest dilution of serum which prevented the growth of virus.

RESULTS

Inactivation of FMD virus with acetylethyleneimine and formalin

The experiments described by Brown & Crick (1959) showed that virus suspensions in tris buffer, pH 7·6, prepared from guinea-pig pad epithelium could be made non-infective for mice and guinea-pigs by incubating with 0·05 % AEI for 4 hr. at 37° C. As virus for the large-scale production of FMD vaccines is most conveniently produced in cultivated cells, the inactivation of virus grown in tissue culture cells was first examined. Whereas 4 hr. was sufficient for the inactivation of virus grown in guinea-pig pad epithelium and suspended in tris buffer, a rather longer period of reaction was required for complete inactivation of both bovine tongue epithelium and pig kidney culture viruses (as tested in mice). This increase in time is probably caused by reaction of the AEI with some of the constituents of the medium in which the cultured cells are maintained; for example, the concentration of amino acids is much greater in tissue culture virus suspensions than in pad epithelium extracts. Dialysis of tissue culture viruses against tris buffer before addition of the AEI increased the subsequent rate of inactivation but the losses of infective virus by adsorption on to the dialysis sac were considered large enough to outweigh the advantages of a shorter inactivation period (see below). On the basis of several experiments, 6 hr. was finally chosen as the inactivation time required for the production of a vaccine which would be innocuous for mice and guinea-pigs. In order to obtain a product which was innocuous for cattle, longer periods of inactivation were required. In five experiments, using six cattle in each experiment, so that a total of 12 ml. of each vaccine was tested, it was found that infective virus was still present after 8 hr. incubation but could not be detected after 12 hr. The results are summarized in Table 1.

The inactivation of FMD virus with 0·05 % formalin at pH 7·6 and 26° C. proceeds rapidly in the initial stages of the reaction, but the rate later decreases and the product is innocuous for mice only after periods greater than 50 hr. In some experiments virus could be detected in mice even after 72 hr. incubation. By adjusting the virus suspension to pH 9 with glycine buffer before adding the formalin, the required incubation period could be reduced to 48 hr. These findings are in general agreement with those of Wesslen & Dinter (1957), who used calf kidney tissue culture cells for detecting the residual virus. However, in all the experiments in which the vaccines were tested for innocuity by intradermal inoculation of cattle tongues, infective virus was detected in suspensions which had been incubated with 0·05 % formalin for periods up to 144 hr., either at pH 7·6 or at pH 9·0. Prior filtration of the virus suspension through a Seitz EK filter had no effect on the result. These experiments are summarized in Table 1.

If the virus is first added to aluminium hydroxide, as in the commercial process, and then incubated with 0·05 % formalin, the product is apparently innocuous for

cattle after 48 hr. It seems likely, from the results described in the previous paragraph, that infective virus will survive this procedure unless the adsorption of FMD virus to aluminium hydroxide renders it more sensitive to the action of formalin.

Table 1. *Inactivation of FMD virus with AEI and formalin*

Virus strain and infectivity (log ID50/ml.)		Inactivation procedure	Proportion of cattle reacting	No. of reaction sites	Interval between inoculation and reaction (days)
997	7·3	0·05% formalin, pH 7·6, 26° C., 114 hr.	1/6	1/120	4
997	7·8	0·05% formalin, pH 7·6, 26° C., 121 hr.	2/6	2/120	3 and 4
997	7·5	0·05% formalin, pH 7·6, 26° C., 144 hr.	2/6	3/120	2 and 5
997	7·5	0·05% formalin, pH 7·6, 26° C., 120 hr.	3/6	4/120	3, 3 and 3
		EK filtered. Then as above	2/6	3/120	3 and 3
997	7·0	0·05% formalin, pH 8·9, 26° C., 72 hr.	6/6	10/120	1, 2, 2, 2, 2, 2
997	6·5	0·05% formalin, pH 8·9, 26° C., 144 hr.	3/6	3/120	3, 4 and 5
997	7·0	0·05% formalin, pH 8·9, 26° C., in presence of $Al(OH)_3$, 72 hr.	0/6	0/120	—
PAK 1	7·4	0·05% formalin, pH 8·9, 26° C., in presence of $Al(OH)_3$, 72 hr.	0/6	0/120	—
997	7·5	0·05% AEI, pH 7·6, 37° C., 8 hr.	3/6	13/120	3, 5 and 5
997	7·0	0·05% AEI, pH 7·6, 37° C., 12 hr.	0/6	0/120	—
		0·05% AEI, pH 7·6, 37° C., 18 hr.	0/6	0/120	—
PAK 1	7·4	0·05% AEI, pH 7·6, 37° C., 12 hr.	0/6	0/120	—
PAK 1	5·4	0·05% AEI, pH 7·6, 37° C., 12 hr.	0/6	0/120	—

Comparison of the potency of AEI and formalin vaccines for guinea-pigs

Before proceeding to experiments in cattle, it was desirable to ascertain whether AEI-inactivated tissue culture vaccine was effective in guinea-pigs. In preliminary experiments it was found that inactivation of the tissue culture virus proceeded more rapidly if it was first dialysed against tris buffer, but this process

led to the loss of about 0·5 log of virus infectivity, presumably as a result of adsorption on to the dialysis casing. This loss of virus was reflected in the loss of potency of the vaccine prepared from the dialysed virus (see Table 3). In subsequent experiments the dialysis step was omitted and the longer inactivation period (6 hr.) was used.

It seemed desirable that the action of the AEI should be stopped after the appropriate incubation period; it was essential, however, that the agent used for 'neutralizing' the AEI should have no adverse effects on the potency of the vaccine.

Table 2. *Neutralization of FMD virus inactivating agents*

Mixture	Infectivity log (mouse ID 50/ml.)
Virus only, 37°/4 hr.	7·0
Virus + 0·05 % AEI, 37°/4 hr.	None detected
Virus + (0·05 % AEI + 2% sodium thiosulphate), 37°/4 hr.	7·1
Virus only, 26°/48 hr.	5·1
Virus + 0·05 % formalin, 26°/48 hr.	1·0
Virus + (0·05 % formalin + sodium bisulphite), 26°/48 hr.	4·8

Table 3. *Comparison of AEI and formalin FMD vaccines (strain 997) in guinea-pigs*

	Guinea-pigs protected by			Neutralization index of 1/20 sera (log ID 50)		
Virus content log (mouse ID 50/dose)	AEI vaccine + thiosulphate	Dialysed AEI vaccine + thiosulphate	Formalin vaccine + bisulphite	AEI vaccine	Dialysed AEI vaccine	Formalin vaccine
6·0	5/5	5/5	5/5	—	—	—
5·5	5/5	2/5	5/5	2·0	1·5	1·9
5·0	5/5	1/5	3/5	2·0	1·0	1·4
4·5	0/5	0/5	0/5	—	—	—

Sodium thiosulphate (2 %) was shown to completely 'neutralize' 0·05 % AEI (Table 2) and it also had no effect on the potency of vaccines prepared by inactivation with AEI (Table 4). Vaccines prepared with formalin were neutralized with excess sodium bisulphite after the inactivation period. The bisulphite had no effect on vaccine potency (Table 4) and is effective in stopping the reaction of formalin with virus (Table 2).

Vaccines were prepared from pig kidney culture virus (strain 997) by inactivation with 0·05 % AEI at 37° C. for 6 hr. or by incubating with 0·05 % formalin for 96 hr. at pH 7·6. An AEI vaccine was also prepared from a sample of the virus which had been dialysed against tris buffer. Inactivation was stopped with sodium thiosulphate and sodium bisulphite respectively, and each vaccine was tested for innocuity in both mice and guinea-pigs. Dilutions of each vaccine were then mixed with aluminium hydroxide and inoculated subcutaneously into guinea-pigs. The

results of the subsequent challenge of the guinea-pigs with virus at 21 days are shown in Table 3, from which it can be seen that the 6 hr. AEI vaccine is slightly superior to the 96 hr. formalin vaccine prepared at pH 7·6. In another experiment with a different virus suspension, the effect of adding thiosulphate and bisulphite to the two vaccines was tested and these results are shown in Table 4. The results of the experiments with the AEI vaccine were considered sufficiently encouraging to warrant their extension to cattle.

Table 4. *Effect of thiosulphate and bisulphite on the potency of FMD vaccines (strain 997) in guinea-pigs*

Virus content log (mouse ID50/dose)	Guinea-pigs protected by			
	AEI vaccine	AEI + thio-sulphate	Formalin vaccine	Formalin + bi-sulphite
6·0	10/10	10/10	10/10	10/10
5·5	10/10	10/10	9/10	10/10
5·0	7/10	9/10	7/10	8/10
4·5	5/10	7/10	4/10	3/10

Comparison of the potency of AEI and formalin vaccines for cattle

As indicated above, it has not been possible in the present experiments to obtain a formalin vaccine which was innocuous for cattle (by intradermal inoculation) unless aluminium hydroxide was added. In consequence, all comparisons in cattle between AEI and formalin vaccines have been made with formalin vaccines prepared in the presence of aluminium hydroxide. The results of one experiment with virus of strain 997, produced in cultivated pig kidney cells, indicate that an AEI vaccine inactivated for 18 hr. is as effective as the conventional formalin vaccine (Table 5).

Table 5. *Comparison of AEI and formalin FMD vaccines (strain 997) in cattle*

Virus content log (mouse ID50/dose)	Cattle protected by vaccines	
	AEI	Formalin
6·5	5/8	3/8
7·0	7/8	7/8
7·5	8/8	6/8

In a second experiment, samples of virus of strain Pak 1 grown in surviving bovine tongue epithelium cells ($10^{7·4}$ mouse ID50/ml.) were inactivated by each method and the levels of specific neutralizing antibody produced in cattle were determined (Fig. 1). The cattle used in this experiment were not challenged because they were required for subsequent studies on the effects of re-vaccination with inactivated FMD vaccines (Hyslop, unpublished observations). The specific antibody levels resulting from the primary response to the two vaccines, however,

again demonstrated the efficacy of AEI as an inactivating agent for the preparation of FMD vaccines.

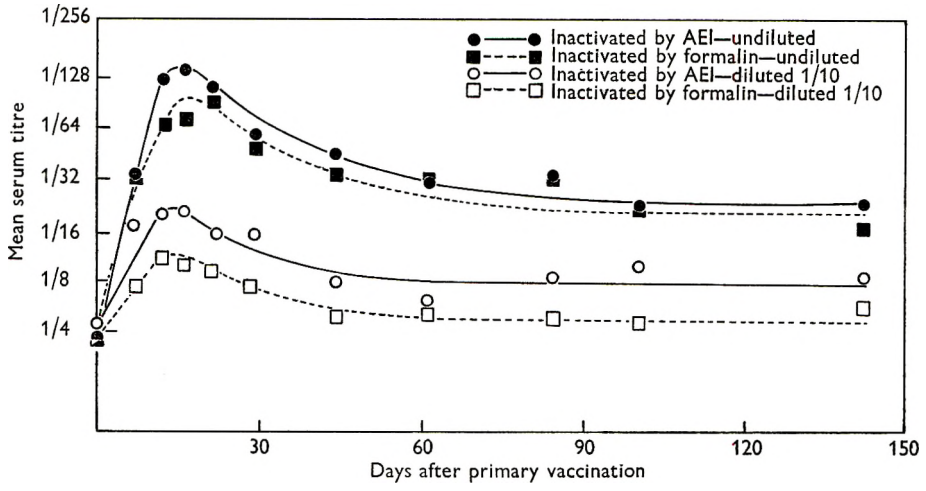


Fig. 1. Mean serum antibody titres of groups of 8 cattle inoculated with $10^{8\cdot6}$ or $10^{7\cdot6}$ ID_{50} of virus treated with AEI for 12 hr. or formalin for 48 hr.

DISCUSSION

The reaction of animal viruses with dilute formalin is a complex process. In common with other viruses, the initial rate of inactivation of FMD virus is extremely rapid but prolonged incubation periods are required to obtain a non-infective product. In normal practice FMD vaccines are produced by inactivation of virus which has been adsorbed on to aluminium hydroxide before or at the time of addition of the dilute formalin. Unless this process of adsorption renders the virus more sensitive to the action of formalin it is certain that these vaccines will contain infective particles at the end of the 48 hr. incubation period. Evidence has, in fact, been produced (Moosbrugger, 1948; Schneider, 1955) that vaccines prepared in the presence of aluminium hydroxide which were innocuous for cattle did contain infective virus particles. In our experiments we have failed to inactivate completely the virus for cattle by incubating the suspensions with 0·05% formalin for periods up to 144 hr.

These experiments also emphasize that vaccines which are to be used in cattle should not be tested for innocuity only in mice or tissue-culture cells. This difference in sensitivity of the three host systems towards formalin-treated virus is interesting in view of their equal sensitivity towards the untreated virus. In this connexion it may be significant that the virus detected by the intradermal inoculation of cattle with suspensions which have been incubated with formalin for long periods produces lesions as late as 5 days after inoculation, whereas the lesions produced by untreated virus generally occur within 24–48 hr. (Table 1).

Incubation with AEI instead of formalin is a more certain method of obtaining a vaccine free from infective virus and the results described above indicate that such vaccines are as potent antigenically as formalin vaccines of the same strain produced by methods used commercially.

SUMMARY

Suspensions of FMD virus treated with 0·05 % formalin at 26° C. for periods up to 144 hr. remained infective for cattle, although the infectivity could not be detected in the presence of aluminium hydroxide. Infectivity was detected in similar virus suspensions which had been treated with 0·05 % AEI at 37° C. for 8 hr. but not in suspensions treated for 12 hr.

Vaccines prepared from these suspensions were antigenically potent and serum neutralization tests demonstrated the development and regression of serum antibody. The AEI vaccines were at least as potent as the corresponding formalin vaccines.

We wish to thank Dr W. B. Martin for supplying us with some of the virus suspensions and Mrs S. P. Carter for carrying out the serum neutralization tests. Mr G. V. Short of Imperial Chemical Industries Ltd. kindly supplied the acetylethyleneimine.

REFERENCES

BROWN, F. & CRICK, J. (1959). Application of agar-gel diffusion analysis to a study of the antigenic structure of inactivated vaccines prepared from the virus of foot-and-mouth disease. *J. Immunol.* **8**, 444.

FRENKEL, H. S. (1951). Research on foot-and-mouth disease. III. The cultivation of the virus on a practical scale in explanations of bovine tongue epithelium. *Amer. J. vet. Res.* **12**, 187.

HENDERSON, W. M. (1952). Significance of tests for non-infectivity of foot-and-mouth disease vaccines. *J. Hyg., Camb.*, **50**, 195.

MARTIN, W. B. & CHAPMAN, W. G. (1961). The tissue culture colour test for assaying the virus and neutralising antibody of foot-and-mouth disease and its application to the measurement of immunity in cattle. *Res. vet. Sci.* **2**, 53.

MOOSBRUGGER, G. A. (1948). Recherches expérimentales sur la fièvre aphteuse. *Schweiz. Arch. Tierheilk.* **90**, 176.

SCHNEIDER, B. (1955). Zur Infektiosität der Maul-und-Klauenseuche Adsorbat-Vakzine. *Mh. Tierheilk.* **7**, 81.

SELLERS, R. F. (1955). Growth and titration of the viruses of foot-and-mouth disease and vesicular stomatitis in kidney monolayer tissue cultures. *Nature, Lond.*, **176**, 547.

SKINNER, H. H. (1951). Propagation of strains of foot-and-mouth disease virus in unweaned white mice. *Proc. R. Soc. Med.* **44**, 1041.

SKINNER, H. H. (1953). One week old white mice as test animals in foot-and-mouth disease research. *Proc. 15th Int. vet. Congr.* **1**, 195.

VALLÉE, H., CARRÉ, H. & RINJARD, J. (1926). Sur l'immunisation anti-aphteuse par le virus formolé. *Rév. gén. méd. vét.* **35**, 129.

WALDMANN, O. & KÖBE, K. (1938). Die aktive Immunisierung des Rindes gegen Maul-und-Klauenseuche. *Berl. tierärztl. Wschr.* **46**, 317.

WESSLEN, T. & DINTER, Z. (1957). The inactivation of foot-and-mouth disease virus by formalin. *Arch. Virusforschung*, **7**, 394.

J. Hyg. Camb. (1963), **61**, 345
Printed in Great Britain

In vitro measurement of the potency of inactivated foot-and-mouth disease virus vaccines

By F. BROWN and J. F. E. NEWMAN
Research Institute (Animal Virus Diseases), Pirbright, Surrey

(*Received* 15 *March* 1963)

INTRODUCTION

An *in vitro* test which measures the potency of inactivated foot-and-mouth disease (FMD) virus vaccines would be of considerable value because the currently available animal potency tests are extremely costly. In addition, the tests are lengthy, requiring 4 weeks to complete, so that any emergency requirements cannot be met with vaccines of known potency unless these have been tested prior to storage.

Randrup (1954) and Brown & Crick (1959) have shown that the 25 mμ component of FMD virus suspensions possesses immunizing properties after suitable inactivation of its infectivity, whereas the accompanying 7 mμ component is not immunogenic. It seemed worthwhile, therefore, to examine the relationship between the amount of the inactivated 25 mμ component in different FMD virus suspensions and the potency of the vaccines prepared from them.

MATERIALS AND METHODS

Virus preparations and titration

Strain 1 (type O) was used in all the experiments described. This is a strain which has been maintained for more than 30 years by passage in guinea-pigs by intradermal inoculation of the hind pads and subsequent harvesting of the pads and vesicle fluid 24 hr. after inoculation. In these experiments the pads and vesicle fluid were harvested separately. Virus suspensions were prepared from the pad epithelium by grinding in a mortar with 0·04 M phosphate buffer, pH 7·6. Mouse virus was obtained by one passage of the guinea-pig vesicle fluid (intraperitoneal inoculation of 10^{-6} dilution) in 7-day-old unweaned mice and the leg muscles were collected from the mice immediately after death. The virus was extracted by grinding the mouse tissue in 0·04 M phosphate buffer, pH 7·6. Virus was also grown in monolayers of pig kidney and baby hamster kidney cells (BHK 21—MacPherson & Stoker, 1962) by infecting each type of monolayer with guinea-pig vesicle fluid and incubating in nutrient medium at 37° C. until the cell sheet was destroyed by the action of the virus. For the pig kidney cells, Earle's saline containing 0·5 % lactalbumin hydrolysate and 0·01 % yeast extract was used; a modified Eagle's medium (MacPherson & Stoker, 1962) was used for the baby hamster kidney cells. The cell debris was removed from the tissue culture harvests by centrifugation at 2000 r.p.m. All the virus titrations were carried out by intraperitoneal inoculation of 7-day-old unweaned mice (Skinner, 1951).

Estimation of complement-fixing antigens

Comparison of the amounts of complement-fixing antigens present in the different suspensions was made by using Brooksby's method (1952), in which the virus and antiserum are allowed to react with different amounts of complement and the 50 % haemolytic end-point calculated by the use of probits.

Treatment of suspensions with Arcton

Virus suspensions were homogenized at 13,000 r.p.m. with one-half volume of Arcton 113 (I.C.I. Ltd.), maintaining the suspension at 0° C. The homogenate was then centrifuged at 2000 r.p.m. and the aqueous layer treated in the same way until homogenization no longer produced an interface between the aqueous and solvent layers.

Inactivation and potency testing

Acetylethyleneimine (I.C.I. Ltd.) was used for virus inactivations (Brown & Crick, 1959). As a standard procedure, all virus suspensions were incubated for 6 hr. at 37° C. with 0·05 % acetylethyleneimine. The reaction was then stopped with 2 % sodium thiosulphate and the preparations tested for innocuity by intraperitoneal inoculation of mice. Appropriate dilutions of the vaccines were then mixed with equal volumes of aluminium hydroxide gel and inoculated subcutaneously into groups of guinea-pigs. Twenty-one days after vaccination, the animals were challenged by intradermal inoculation of one hind pad with 10^4 ID 50 of infective virus (prepared from infected guinea-pig pads) and the animals examined daily for 10 days for the development of lesions on the feet and tongue. From the number of animals showing lesions on the feet and tongue, the dilution of vaccine which protected 50 % of the animals from generalized infection was calculated.

RESULTS

Concentration of 25 mμ component in different virus suspensions

Since previous work by Randrup (1954) and Brown & Crick (1959) has indicated the importance of the 25 mμ component in FMD virus vaccines, a method was sought which would allow the rapid and accurate estimation of this component in several virus suspensions. The relative amounts of the 25 mμ component in five different virus suspensions, prepared from guinea-pig vesicle fluid, guinea-pig pad epithelium, infected mice and cultivated pig kidney and baby hamster kidney cells were compared by estimating the amount of complement which they fixed after treatment with Arcton 113. This procedure removes the 7 mμ component of FMD virus suspensions without lowering their infectivity (Mussgay, 1959; Brown & Cartwright, 1960). The data in Table 1 show that the proportion of the complement-fixing activity due to the 25 mμ component of the virus is about 50 % for the vesicle fluid and tissue culture viruses, but considerably lower for the pad epithelium and mouse tissue suspensions.

Table 1. *Concentration of* 25 mμ *component in FMD virus suspensions*

Virus source	Complement-fixing activity (ml. 1/30 C′/ml. antigen)	
	Initial suspension	Arcton-treated suspension
Vesicle fluid	144·0	72·0
Pad epithelium	4·12	0·80
Mouse muscle	15·4	2·0
Pig kidney cells	0·23	0·11
Baby hamster kidney cells	1·23	0·49

Comparison of potencies of different vaccines

The potencies of vaccines prepared from the five different sources of the virus were compared in experiments with guinea-pigs. The results of these experiments show that the amount of 25 mμ component required to protect 50% of the vaccinated animals against generalized infection was fairly constant for different batches of a given source of virus, but varied considerably between sources (Table 2). The values obtained for any of the five individual sources of virus used here varied by a factor less than 3. The difference in the amounts of 25 mμ component in the guinea-pig and tissue culture vaccines which were required for 50% protection was greater than this factor, suggesting a difference between the virus suspensions which was not measurable by this test.

Table 2. *Potency of FMD vaccines prepared from different sources of the virus*

Source	Complement-fixing activity of 25 mμ component (ml. 1/30 C′/ml.)	Amount of 25 mμ component protecting 50% of animals (ml. 1/30 C′/ml.)
Guinea-pig vesicle fluid	38·5	0·08
	60·0	0·17
	72·0	0·11
	55·0	0·11
Guinea-pig pad epithelium	1·6	0·16
	0·60	0·30
	1·1	0·20
Mouse muscle	0·63	0·08
	1·1	0·16
	2·3	0·07
	1·3	0·06
Pig kidney cells	0·095	0·02
	0·05	0·01
	0·11	0·01
	0·045	0·015
Baby hamster kidney cells	0·49	0·01
	0·32	0·01
	1·1	0·02

Physical properties of the immunizing antigen

In view of the differences between the amounts of 25 mμ component (measured by complement-fixation tests) in the different suspensions which were required to produce immunity, the nature of the immunizing antigen in guinea-pig vesicle fluid and in pig kidney tissue culture virus was examined in more detail. With each suspension, the immunizing antigen was deposited by centrifuging at 30,000 r.p.m. for 150 min. (Spinco L 40 head), survived extraction with Arcton 113 (which removes all the 7 mμ component of the virus), and was unaffected by incubation at 25° C. or 37° C. for periods which reduced the infectivity to less than 0·1 % of the initial value. The immunizing antigen was completely destroyed on heating to 56° C. and considerably reduced by lowering the pH to 6·5. These properties are also possessed by the 25 mμ infective component of the virus, thus confirming the earlier conclusion reached for guinea-pig vesicle fluid that the immunizing antigen is associated with the 25 mμ component of the virus.

DISCUSSION

The lengthy and costly tests in cattle which are currently used to assess the potency of inactivated FMD vaccines make it worthwhile to explore the possibility of devising an *in vitro* test for this purpose. The prime requirement of such a test is that it should correlate reliably with the *in vivo* test. A serological test of this sort has been devised by Beale (1961) for polio vaccines.

The method most extensively used for the production of commercial FMD vaccines is based on the inactivation of the virus following its adsorption to aluminium hydroxide gel. *In vitro* testing of such vaccines would require prior elution of the inactivated virus from the aluminium hydroxide. Elution is difficult so that efforts to correlate the potency of FMD vaccines with other properties of the virus suspensions from which they were prepared must, at this stage, be made with the virus suspensions before inactivation.

In the experiments described here, the physical properties of the immunizing antigen have been studied in some detail. In accordance with the previous reports of Randrup (1954) and Brown & Crick (1959) the 25 mμ component of the virus has been shown to be immunogenic. Further, the 25 mμ component and immunizing antigen appear to behave identically under a variety of conditions, suggesting that the entire immunogenicity is associated with the 25 mμ component. Of particular interest in connexion with the growth of virus for the preparation of FMD vaccines is the fact that the immunizing properties of the virus suspensions are unaltered by incubation at 37° C. for periods long enough to reduce their infectivity titre by more than 99 %. This means that the stability of the immunizing antigen at 37° C. is much greater than that of the infective component of the virus. Brown, Cartwright & Stewart (1963) have shown that the effect of incubating FMD virus suspensions at 37° C. is to destroy the infectivity of the ribonucleic acid component of the virus without significantly altering the viral protein.

When FMD virus is grown in cultured cells at 37° C., virus released into the

medium during the early part of the growth cycle will decrease in infectivity (*ca.* 1 log in 8 hr.) although its immunogenicity will be unaffected. Unless the virus is released from all the susceptible cells over a short time interval, however, the times at which maximum infectivity and immunogenicity are reached will not coincide. This has been clearly demonstrated by Henderson (1953) and Ubertini, Nardelli, Barei & Santen (1956) in experiments with virus grown in surviving

Table 3. *Relationship between infectivity, complement-fixing activity and immunogenicity of two FMD virus suspensions*

Treatment or fraction	Infectivity (log ID50/ml.)	Complement-fixing activity (ml. 1/30 C′ fixed by 1 ml.)	Dilution of vaccine protecting 50% of animals
Initial virus (guinea-pig vesicle fluid)	9·2	144·0	1/750
Heated at 25° C. for 72 hr.	6·0	140·1	1/500
Heated at 37° C. for 48 hr.	3·2	144·0	1/750
Acidified to pH 6·5	5·9	267·2	1/20
Heated at 56° C. for 0·5 hr.	4·4	202·0	No protection with 1/5
Arcton-extracted	9·2	72·5	1/500
Fractions from centrifuge 30,000 r.p.m. for 1 hr.			
Top 7 ml.	5·5	47·5	1/5
Bottom 4 ml.	6·3	27·3	1/20
Pellet	8·9	93·5	1/350
Initial virus (pig kidney tissue culture suspension)	6·0	0·23	1/10
Heated at 37° C. for 24 hr.	3·0	0·22	1/5
Heated at 56° C. for 0·5 hr.	< 2·0	0·34	No protection with 1/1
Acidified to pH 6·5	5·0	0·37	No protection with 1/1
Arcton-extracted	6·0	0·11	1/10
Fractions from centrifuge 30,000 r.p.m. for 1 hr.			
Top 7 ml.	2·8	0·04	No protection with 1/1
Bottom 4 ml.	4·2	0·06	No protection with 1/1
Pellet	5·9	0·10	1/4

tongue epithelium fragments. Experiments at this Institute with several strains of the virus have shown that it is possible to assess the potency of a vaccine from its infectivity titre if the growth cycle of the particular strain is well characterized (Henderson & Galloway, unpublished observations). Differences in the multiplication of different virus strains, even in the same tissue system, make it essential to study the growth cycle under precisely controlled conditions before an assessment of its potency can be made from its infectivity titre. Even with these precautions, a relationship between infectivity and potency which is applicable to all virus strains grown in a variety of tissues or cells would be difficult to establish.

It is known that the complement-fixing activity of FMD virus suspensions which have been extracted with Arcton is a measure of both the infective and non-infective 25 mμ components of the virus. Consequently, the relationship between the complement-fixing activity of the 25 mμ component in several virus suspensions and their potencies as vaccines for guinea-pigs has been examined. For different preparations of virus from one kind of animal tissue or cell, the amount of 25 mμ component required to protect 50 % of the guinea-pigs varied by less than threefold. Larger differences were obtained, however, between the various sources used here. For example, the 50 % protective dose of 25 mμ component was about 10 times greater for guinea-pig vesicle fluid than for pig kidney or baby hamster kidney virus. This may be due to masking of the 25 mμ component in the tissue culture viruses by a cellular component. Masking of this sort is known to occur with FMD virus (Brown, Cartwright & Stewart, 1962) and also with poliomyelitis virus (Holland & McLaren, 1959). While, therefore, the complement-fixing activity of the 25 mμ component of suspensions can be used as an index of their potency as vaccines if they are all derived from similar animal or culture systems, the relation between vaccines prepared from different cell systems has still to be determined.

SUMMARY

Inactivated vaccines have been prepared from one strain of FMD virus grown in guinea-pig pad epithelium, unweaned mice and cultured pig kidney and baby hamster kidney cells. The potencies of these vaccines in protecting guinea-pigs against challenge with inoculated infective virus of the same strain have been compared and related to the amounts of 25 mμ component present in the different virus suspensions. Although it was possible to obtain a relationship between the content of 25 mμ component and potency for an individual source of virus, this relationship does not hold for all the different sources of virus used. It is suggested that the reason for this failure is the partial masking of the 25 mμ component by a cell constituent present in some of the virus suspensions so that the component is incompletely estimated by the complement-fixation test.

REFERENCES

BEALE, A. J. (1961). The D-antigen content in poliovaccine as a measure of potency. *Lancet*, ii, 1166.

BROOKSBY, J. B. (1952). The technique of complement-fixation in foot-and-mouth disease research. *Agric. Res. Coun. Rep. Ser.* no. 12.

BROWN, F. & CARTWRIGHT, B. (1960). Purification of the virus of foot-and-mouth disease by fluorocarbon treatment and its dissociation from neutralizing antibody. *J. Immunol.* **85**, 309.

BROWN, F., CARTWRIGHT, B. & STEWART, D. L. (1962). Further studies on the infection of pig-kidney cells by foot-and-mouth disease virus. *Biochim. Biophys. Acta*, **55**, 768.

BROWN, F., CARTWRIGHT, B. & STEWART, D. L. (1963). The effect of various inactivating agents on the viral and ribonucleic acid infectivities of foot-and-mouth disease virus and on its attachment to susceptible cells. *J. gen. Microbiol.* **31**, 444.

BROWN, F. & CRICK, J. (1959). Application of agar-gel diffusion analysis to a study of the antigenic structure of inactivated vaccines prepared from the virus of foot-and-mouth disease. *J. Immunol.* **82**, 444.

HENDERSON, W. M. (1953). The use of virus culture in foot-and-mouth disease research. *Proc. 15th Int. vet. Congr.* **1**, 206.

HOLLAND, J. J. & MCLAREN, L. C. (1959). The mammalian cell–virus relationship. II. Adsorption, reception, and eclipse of polio-virus by HeLa cells. *J. exp. Med.* **109**, 487.

MACPHERSON, I. A. & STOKER, M. G. P. (1962). Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology*, **16**, 147.

MUSSGAY, M. (1959). Trennung zweier spezifischer Einheiten des Virus der Maul-und-Klauenseuche mit einem Fluor-Kohlenwasserstoff. *Z. Hyg. InfektKr.* **146**, 48.

RANDRUP, A. (1954). On the stability of bovine foot-and-mouth disease virus dependent on pH. Investigations on the complement fixing and the immunising antigen as well as on the Infective agent. *Acta path. microbiol. Scand.* **35**, 388.

SKINNER, H. H. (1951). Propagation of strains of foot-and-mouth disease virus in unweaned white mice. *Proc. R. Soc. Med.* **44**, 1041.

UBERTINI, B., NARDELLI, L., BAREI, S. & SANTEN, G. (1956). Études sur la culture *in vitro* du virus aphteux selon Frenkel. *Zbl. vet. Med.* **3**, 419.

J. Hyg. Camb. (1963), **61**, 353
Printed in Great Britain

Comparative studies on *Salmonella typhi* grown *in vivo* and *in vitro*

III. The immunizing potencies of acetone-killed vaccines prepared from *in vivo*- and *in vitro*-grown bacteria and the immunizing potency of substances isolated from infected organs

By A. L. OLITZKI and DINA GODINGER

Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received* 20 *March* 1963)

In previous experiments of Olitzki & Godinger (1963) it was demonstrated that *Salmonella typhi* Ty2 grown *in vivo* was more virulent for mice than its corresponding culture grown *in vitro*. These authors also showed that extracts of infected organs acted as infection-promoting substances. *In vitro* experiments of Olitzki & Kaplan (1963) proved that the majority of organ extracts of mice infected with strain Ty2 inhibited markedly the bactericidal action of serum on *S. typhi*. The experiments described below were carried out in order to examine whether the *in vivo*-grown *S. typhi* strain Ty2, employed for the preparation of an acetone-killed vaccine, possesses a higher immunizing potency than its corresponding culture grown *in vitro*. Experiments were also carried out in order to show whether organ extracts of infected animals contained immunizing antigens similar to those of the intact bacteria isolated from the infected organs.

METHODS

The immunization and challenge procedures varied according to the conditions of the individual experiment. The details will be described below. *S. typhi*, strain Ty2, was used in all experiments for the preparation of the vaccines and for the challenge dose as well. In all experiments white mice of both sexes, 3–4 weeks old at the start of the experiment, and weighing on an average 20 g. were used. The interval between the immunizing injection and the challenge dose was 14 days. Both injections were given intraperitoneally. The total volumes of the immunizing material and the challenge dose was in all experiments 0·5 ml. The challenge dose contained in the majority of the experiments 2×10^8 bacteria with the exception of one experiment, presented in Table 2, where $2{\cdot}5 \times 10^8$ were given.

In the first group of experiments we compared the immunizing power of strain Ty2 subcultured 20 times on trypticase-agar (Difco) and harvested after an incubation of 20 hr. at 37° C. with the immunizing power of bacteria of the same parent strain after ten intraperitoneal passages *in vivo* in mice or guinea-pigs and harvested from the peritoneal fluid 18 hr. after the onset of the infection. The infecting dose was 2×10^8 bacteria. The *in vivo*-grown bacteria were carefully freed

from all cell debris by repeated slow centrifugation. Then the bacteria were spun down by centrifugation at 10,000 rev./min. and the living bacterial count of both vaccines was determined by plating, before drying them in acetone. Before each experiment the O-agglutinability of both the *in vitro*- and the *in vivo*-grown bacteria was tested by an anti-O 901 immune serum. The titre for the strain O 901 was 1/10,000, for Ty 2 grown *in vitro* 1/80, and for Ty 2 grown *in vivo* 1/40. From the dried material suspensions were prepared which contained, as calculated from the living count, 6×10^9 bacteria/ml. Groups of mice received intraperitoneally 0·5 ml. of 5-fold dilutions starting with dilution 1/80 which contained $3{\cdot}75 \times 10^7$ micro-organisms/0·5 ml. After an interval of 14 days the mice were challenged with 2×10^8 bacteria of strain Ty 2 grown *in vitro*.

In a second group of experiments we examined the immunizing power of extracts of infected organs. The technique of the preparation of organ extracts was the same as that described by Olitzki & Godinger (1963). The interval between administration of the vaccinating material and the challenge dose was again 14 days. The way of injection and the challenge dose were the same as in the previous experiment with intact bacteria. In the first experiment, in which we worked with untreated extracts, we had to exclude the possibility that residual living bacteria could exert some immunizing effect. For this purpose, the survivors of primary infections with different quantities of living bacteria, suspended in saline or in organ extracts, were challenged in the same way as those treated with organ extracts. However, later it became evident that organ extracts heated at 50° C. for 1 hr. which contained only dead bacteria and also those filtered through Seitz filter SS 1 and freed from living and dead bacteria as well exerted marked immunizing effects. Thus, the participation of residual living or dead bacteria could be completely excluded.

RESULTS

Immunization experiments with vaccines prepared from in vitro- *and* in vivo-*grown* S. typhi *strain* Ty 2

The results of two vaccination experiments are summarized in Table 1. Although the non-protective dilutions of the vaccines were not reached, Table 1 shows that $1{\cdot}5 \times 10^6$ *in vitro*-grown bacteria were able to protect only 68 % of the animals, while the same number of *in vivo*-grown bacteria afforded complete protection.

We therefore extended our experiments and examined the immunizing power of bacteria grown in the abdominal cavity and in the spleen of mice and guinea-pigs as well. The acetone-dried bacteria taken from these environments proved to be non-toxic, when injected in quantities of $7{\cdot}5 \times 10^7$ or less. No deaths were observed within an observation time of 14 days after the vaccination had taken place. The results of these experiments are summarized in Table 2. Although the challenge dose was somewhat higher than in the previous experiments ($2{\cdot}5 \times 10^8$ bacteria grown *in vitro*) the differences in the potencies of the vaccines were similar to those presented in Table 1. $1{\cdot}5 \times 10^6$ bacteria grown in the mouse abdomen and killed by acetone were able to protect 16 out of 20 mice (= 80 %), while the same

quantity of bacteria grown *in vitro* or *in vivo* in the spleens of mice or in guinea-pigs were unable to protect.

Table 1. *Vaccination of mice with* in vitro- *and* in vivo-*grown* S. typhi, *strain* Ty 2 *acetone dried and resuspended in saline*

(Challenge dose 2×10^8 bacteria.)

Expt.	Vaccine prepared from strain Ty 2 grown	Bacterial count in the vaccine	Survivors after challenge in groups of 10	Survivors after challenge in groups of 15
1	*In vitro*	$3{\cdot}75 \times 10^7$	10	—
		$7{\cdot}50 \times 10^6$	10	—
		$1{\cdot}50 \times 10^6$	6	—
	In vivo	$3{\cdot}75 \times 10^7$	10	—
		$7{\cdot}50 \times 10^6$	10	—
		$1{\cdot}50 \times 10^6$	10	—
	Controls non-vaccinated		0	—
2	*In vitro*	$7{\cdot}50 \times 10^6$	—	15
		$1{\cdot}50 \times 10^6$	—	11
		$3{\cdot}00 \times 10^5$	—	10
	In vivo	$7{\cdot}50 \times 10^6$	—	15
		$1{\cdot}50 \times 10^6$	—	15
		$3{\cdot}00 \times 10^5$	—	13
	Controls non-vaccinated	—	—	0

Table 2. *Vaccination of mice with* in vitro- *and* in vivo-*grown* S. typhi

(Challenge dose $2{\cdot}5 \times 10^8$ bacteria grown *in vitro*.)

Vaccine from strain Ty 2 grown	No. of survivors in groups of 20: Vaccinated with acetone-killed micro-organisms $3{\cdot}75 \times 10^7$	$7{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^6$	$3{\cdot}0 \times 10^5$	Non-vaccinated
On trypticase agar	12	1	2	0	—
In mouse spleen	12	3	0	0	—
In mouse abdomen	18	16	16	4	—
In guinea-pig spleen	17	6	1	0	—
In guinea-pig abdomen	19	16	0	0	—
Control	—	—	—	—	0

Furthermore, Table 2 shows that there was also a marked effect exerted by bacteria grown in the abdominal cavity of guinea-pigs. $7{\cdot}5 \times 10^6$ bacteria grown in abdominal cavities of guinea-pigs and mice as well protected 16 out of 20 mice (= 80 %), while the protective effect of $7{\cdot}5 \times 10^6$ *in vitro*-grown bacteria was almost zero and that of the spleen-grown bacteria not higher than 30 %.

If we summarize the results of this experiment we find for the four doses of the intraperitoneally grown bacteria (from guinea-pigs or mice) the following percentages of survivors: 92·5, 80·0, 40·0 and 10·0. The corresponding percentages of

all the other groups are: 68·3, 16·7, 5·0 and zero. The average ED 50 of the first group of vaccines was according to this result about 3×10^6 and of the second $1{\cdot}7 \times 10^7$.

In the following experiment we investigated the immunizing potency of the *in vivo*- and *in vitro*-grown strains against challenge doses of both the *in vivo*- and the *in vitro*-grown strains. The *in vitro*-grown bacteria used for vaccine preparation and challenge doses were subcultured 20 times on trypticase agar, the *in vivo*-grown bacteria used for both purposes were subcultured 10 times in the peritoneal cavities of mice. The results of this experiment are summarized in Table 3.

Table 3. *The immunizing potency of* in vitro- *and* in vivo-*grown* S. typhi *strain* Ty 2 *against* in vitro- *and* in vivo-*grown challenge doses*

(All *in vivo*-grown bacteria were harvested from the abdominal cavities of mice. Challenge dose 2×10^8.)

Immunizing dose	No. of survivors in groups of 10 mice after vaccination with vaccine grown			
	In vitro and challenge with bacteria grown		*In vivo* and challenge with bacteria grown	
	In vitro	*In vivo*	*In vitro*	*In vivo*
$1{\cdot}5 \times 10^8$	10	5	10	10
$3{\cdot}0 \times 10^7$	6	1	10	9
$6{\cdot}0 \times 10^6$	1	0	9	5
$1{\cdot}2 \times 10^6$	0	0	7	2
$2{\cdot}4 \times 10^5$	0	0	5	2
$4{\cdot}8 \times 10^4$	0	0	2	0
$9{\cdot}6 \times 10^3$	0	0	0	0
Control non-vaccinated	0	0	—	—

In order to understand the results of this experiment, we have to take into consideration that 2×10^8 bacteria grown *in vitro* represented somewhat more than one lethal dose, while the lethal dose of the *in vivo*-grown strain was $1{\cdot}6 \times 10^6$. The challenge dose, 2×10^8, of the *in vivo*-grown strain represented, therefore, more than 100 lethal doses. The table shows that the *in vitro*-grown strain yielded a relatively weak vaccine. About 3×10^7 micro-organisms were required to afford a 50 % protection against the *in vitro*-grown and $1{\cdot}5 \times 10^8$ to afford a 50 % protection against the *in vivo*-grown challenge organisms. The corresponding quantities of the *in vivo*-grown bacteria were much lower: $2{\cdot}4 \times 10^5$ and $6{\cdot}0 \times 10^6$. Therefore, if we gave the *in vitro*-grown micro-organisms as challenge, the *in vivo*-grown vaccine was about 100 times more effective than the *in vitro*-grown vaccine. However, if we gave the *in vivo*-grown challenge the *in vivo*-grown vaccine was only 25 times more effective than the *in vitro*-grown vaccine.

We also compared the effect of the potency of the bacteria grown in the peritoneal cavity with those grown in the spleen against both types of challenge doses, *in vitro* and *in vivo*. The results are summarized in Table 4.

Table 4 shows that the intraperitoneally grown bacteria immunize better than

the spleen-grown ones. With the intraperitoneally grown vaccine, $1{\cdot}2 \times 10^6$ organisms gave 100 % protection against the *in vitro*-grown challenge, but $6{\cdot}0 \times 10^6$ organisms were required for protection against the *in vivo*-grown challenge. The respective 100 % protecting doses of the spleen-grown vaccine were $3{\cdot}0 \times 10^7$ and $1{\cdot}5 \times 10^8$. These results, together with those presented in Table 3, indicated that the vaccinating potency of spleen-grown bacteria was lower than that of the intraperitoneally grown bacteria. However, the potencies of both vaccines were higher than those of the *in vitro*-grown micro-organisms.

Table 4. *The immunizing potency of two* in vivo*-grown and acetone-killed* S. typhi *vaccines, one grown in the spleen and the other in the abdominal cavity, against challenge by* in vitro *and* in vivo *intra-abdominally grown bacteria derived from the same parent strain*

(Challenge dose 2×10^8.)

Bacterial count of the vaccine	No. of survivors in groups of 10 mice after vaccination with *S. typhi*, strain Ty 2, grown in: The spleen and challenged by bacteria grown		The abdominal cavity and challenged by bacteria grown	
	In vitro	*In vivo*	*In vitro*	*In vivo*
$1{\cdot}5 \times 10^8$	10	10	10	10
$3{\cdot}0 \times 10^7$	10	8	10	10
$6{\cdot}0 \times 10^6$	3	0	10	10
$1{\cdot}2 \times 10^6$	1	0	10	6
$2{\cdot}4 \times 10^5$	1	0	7	0
$4{\cdot}8 \times 10^4$	0	0	0	0
Controls non-vaccinated	0	0	—	—

Immunization experiments with extracts from infected organs

In order to determine whether extracts of infected organs possessed any immunizing potency, we had to exclude the possibility that residual living or dead bacteria, remaining in the extracts after centrifugation, could exert some immunizing effect. Table 5 demonstrates immunity resulting from a primary infection with different quantities of living bacteria suspended in saline or in organ extracts. The animals which received higher inocula of strain Ty 2 died. When the survivors were challenged 14 days later with 2×10^8 bacteria of strain Ty 2 then the majority of those animals survived which had received 2×10^5 or more living bacteria suspended in saline, 2×10^2 or more bacteria suspended in liver extract and 2×10^4 or more bacteria suspended in spleen extract.

The results of the experiment presented in Table 6 show that extracts of infected liver, and spleen and the peritoneal fluid of infected animals exerted some immunizing effect. However, the control experiment carried out simultaneously and presented in Table 7 showed that organ extracts from non-infected animals also exerted a certain immunizing effect, when living bacteria were added to them in such quantities which, when suspended in saline, were unable to immunize. The

minimal quantity of living bacteria able to immunize when injected intra-abdominally together with liver extracts was 10^2 and together with spleen extracts was 10^4. Such a bacterial inoculum when injected together with normal organ extracts was apparently able to multiply *in vivo* and to reach such a high antigen concentration that a solid immunity was established. The control mixtures which contained normal extracts heated at 50° C. or exposed to chloramphenicol together with the added bacteria exerted also some immunizing effects, but the sterility controls showed that they still contained some living bacteria.

Table 5. *The immunity of the survivors from a preceding intraperitoneal infection with* in vitro-*grown* S. typhi *strain* Ty 2 *after a second infection with* 2×10^8 *bacteria of the same strain grown* in vitro

No. of infecting bacteria	Survivors after a second injection in groups surviving after a first injection of bacteria				
		With extract prepared from			
		Liver		Spleen	
	Without extracts	Normal	Infected	Normal	Infected
2×10^8	0/0	—	—	0/0	0/0
2×10^7	1/1	—	—	1/1	1/1
2×10^6	2/3	0/0	0/0	3/3	3/3
2×10^5	4/4	1/1	1/1	5/5	5/5
2×10^4	0/5	2/2	1/1	3/5	5/5
2×10^3	0/5	5/5	3/3	1/5	4/5
2×10^2	—	5/5	5/5	1/5	3/5
Control untreated	0/10	—	—	—	—

The peritoneal fluid behaved differently. When it was taken from infected mice, and the bacteria removed by high-speed centrifugation, it exerted a marked immunizing effect. When it was taken from normal mice and bacteria were added in concentrations up to $10^4/0{\cdot}5$ ml. no immunizing effects were observed. This result indicated that residual bacteria present in the peritoneal fluid after centrifugation were not identical with the immunizing agent involved.

We continued, therefore, the immunization experiments with infected peritoneal fluids and spleens of guinea-pigs which yielded greater quantities of immunizing material. When these products were taken from normal animals and injected together with small quantities of living bacteria they did not show any immunizing effect.

The spleen of each guinea-pig was suspended in 5 ml. of saline and the peritoneal fluid taken up in 10 ml. saline. The number of bacteria before heating at 50° C. for 60 min. was in the spleen extract 2×10^4/ml. and in the peritoneal fluid 10^3/ml.

The experiment presented in Table 8 shows that the peritoneal fluid of guinea-pigs possesses a powerful immunizing potency which could not be ascribed to residual bacteria, since it retained its immunizing power after it was freed from bacteria by filtration through Seitz filter SS 1.

We tried therefore to isolate this agent by precipitation with 25, 40 and 66·5 % of ethanol. The substances precipitated after each addition of ethanol were dried and then taken up in saline at a concentration of 20 mg./ml. When varying quantities of substances were injected into mice and the mice challenged with 2×10^8 bacteria of strain Ty 2 then the results were obtained, which are presented in Table 9.

Table 6. *Immunization experiment with extracts from infected organs*

(Total volume 0·5 ml. Challenge dose 2×10^8 micro-organisms of strain Ty 2 grown *in vitro*.)

Antigen			Living bacteria present in the vaccine	Survivors in groups of 10 mice after injection of the	
				Immunizing antigen	Challenge dose
Preparations	Origin	Dilution			
Disintegration and centrifugation	Liver	Undiluted	15000	4	4
		1/2	7500	9	5
		1/5	3000	10	2
	Spleen	Undiluted	3000	10	10
		1/2	1500	10	1
		1/5	600	10	1
	Peritoneal fluid	Undiluted	14000	10	10
		1/2	7000	10	0
		1/5	2800	10	0
As above then kept for 1 hr. at 50° C.	Liver	Undiluted	0	10	10
		1/2	0	10	5
		1/5	0	10	3
	Spleen	Undiluted	0	10	10
		1/2	0	10	3
		1/5	0	10	0
	Peritoneal fluid	Undiluted	0	10	10
		1/2	0	10	2
		1/5	0	10	0
As above then kept for 18 hr. at 37° C. with 1 mg. chloramphenicol/ml.	Liver	Undiluted	1000	10	10
		1/2	100	10	4
		1/5	40	10	1
	Spleen	Undiluted	50	10	10
		1/2	25	10	4
		1/5	10	10	0
	Peritoneal fluid	Undiluted	0	10	10
		1/2	0	10	0
		1/5	0	10	0
Controls, untreated					0

As control for this experiment we used the original infected peritoneal fluid which allowed, in groups of five mice, in the dilutions 1/10, 1/20 and 1/40, the respective survival of 5, 3 and 1 mice, while in the untreated group no mice survived.

Table 9 shows that the fraction precipitated by 40 % ethanol was the most active one and 0·25 mg. still prevented the death of three out of five mice. The fraction precipitated with 66 % ethanol was almost inactive and protected only when injected in

quantities of 4·0–20·0 mg. The supernatant fluid which remained after the ethanol precipitation and evaporation of the ethanol was inactive and all mice in groups of five died after treatment with 1·0 and 0·5 ml. of the supernatant fluid and infection with 2×10^8 *in vivo*-grown bacteria of strain Ty 2. Also the protein precipitated from this fraction by saturation with ammonium sulphate did not exhibit any immunogenic properties. All fractions proved to be non-toxic and no deaths occurred after the injection of 10·0 and 20·0 mg of any fraction.

Table 7. *Control experiments for the experiment shown in Table* 6

(Immunization of mice with extracts of non-infected organs with addition of living or killed bacteria. Organ extracts undiluted. Total volume 0·5 ml. Challenge dose: 2×10^8 bacteria of strain Ty 2 grown *in vitro*.)

Antigens			Survivors in groups of 5 mice	
Origin of antigen	Bacteria added (strain Ty 2)	Further treatment	After vaccination	After challenge dose
Normal liver	None	None	10	2
	10^2		10	8
	10^3		8	8
Normal spleen	None	None	10	0
	10^2		10	0
	10^3		10	0
	10^4		10	4
Normal peritoneal fluid	None	None	10	0
	10^2		10	0
	10^3		10	0
	10^4		10	0
Normal liver	None	1 hr., 50° C.	10	0
	10^2		10	2
	10^3		10	6
	10^4		10	8
Normal liver	None	Chloramphenicol,	10	0
	10^2	1 mg./ml., 18 hr.,	10	2
	10^3	37° C.	10	8
	10^4		10	8
Extract not added	10^2		10	0
	10^3		10	0
	10^4		10	0
Controls untreated	—	—	—	0

Differences in the chemical and physical properties of in vivo- *and* in vitro-*grown bacteria*

The *in vivo*-grown bacteria differ from the *in vitro*-grown ones in their optical density, weight, nitrogen and protein content as summarized in Table 10.

All these data were obtained after careful removal of cell debris until microscopical examination did not reveal any stainable structures beside bacteria. However, these differences, mainly those between the *in vitro*-grown bacteria and those grown in the abdominal cavity, were not sufficiently great to explain the

Table 8. *Immunization experiment with extracts of spleen and peritoneal fluid of infected and non-infected guinea-pigs, heated at* 50° C.

(Total volume 0·5 ml. Challenge dose 2×10^8 bacteria of strain Ty 2.)

Antigen from guinea-pig	Dilution	Living bacteria present in vaccine before heating	Survivors in groups of 5 mice after injection of strain Ty 2 grown	
			In vitro	*In vivo*
Infected spleen	Undiluted	1000	5	3
	1/4	250	5	0
	1/16	60	2	0
Non-infected spleen	Undiluted	0	0	0
	1/4	0	0	0
	1/16	0	0	0
Infected peritoneal fluid	Undiluted	50	5	5
	1/4	12	5	5
	1/16	3	5	5
Non-infected peritoneal fluid	Undiluted	0	1	0
	1/4	0	0	0
	1/16	0	0	0
Infected peritoneal fluid passed through Seitz filter	1/7·5	0	5	—
	1/15	0	4	—
	1/30	0	2	—
Controls non-vaccinated	—	—	0	0

Table 9. *The immunizing properties of three fractions precipitated from the peritoneal fluid of infected guinea-pigs by addition of* 25, 40 *and* 66 % *ethanol, compared with those of the original peritoneal fluid*

Injected dilutions of the original peritoneal fluid (volume 0·5 ml.)	Injected quantity of each fraction (mg.)	No. of survivors in groups of 5 mice infected with 2×10^8 *in vitro*-grown bacteria after immunization with			
		Fractions precipitated with ethanol			Original infected peritoneal fluid heated at 50° C., 60 min.
		25 %	40 %	66 %	
—	2·0	4	5	1	—
—	1·0	5	5	0	—
—	0·5	4	4	0	—
—	0·25	0	3	0	—
1/10	—	—	—	—	5
1/20	—	—	—	—	3
1/40	—	—	—	—	1

differences in immunological activity. The differences in the optical density were 2·6-fold, the differences in the weight 4-fold and the differences in the nitrogen content 1·5-fold, while the differences in the immunizing potencies were 25-fold against the *in vivo* challenge and 100-fold against the *in vitro* challenge as shown in Table 3. The morphological differences between the *in vivo*- and *in vitro*-grown

bacteria were as follows: the average length of the *in vivo*-grown bacteria, taken from the peritoneal fluid 18 hr. after the onset of infection, was 1·50 μ, 91·8 % of them varying in their length between 0·5 and 2·0 μ and only 8·2 % representing elongated forms varying from 2·5 to 6·0 μ. The average length of the *in vitro*-grown bacteria from an 18 hr. agar culture was 1·74 μ, only 81·1 % of them varied in their length between 0·5 and 2·0 μ, and 18·9 % represented elongated forms varying from 2·5 to 7·0 μ. According to this finding a higher weight of the *in vitro*-grown bacteria should be expected, while in reality the *in vivo*-grown bacteria weighed more. This phenomenon may be explained by the temporary absorption of host-substances during the *in vivo* growth, which was described in the previous communication of Olitzki & Godinger (1963).

Table 10. *Some properties of* in vitro- *and* in vivo-*grown* S. typhi, *strain* Ty 2

	6×10^2 bacteria/ml. of strain Ty 2 grown		
		In vivo in mice	
Properties	*In vitro*	Spleen	Abdominal cavity
Optical density*	0·72	3·76	1·88
Dry weight (mg.)	0·66	11·00	2·66
Nitrogen content (μg.)	16·0	130·0	24·4
Protein content (mg.)	0·10	0·81	0·15

* Wavelength, 5500 Å.

DISCUSSION

In the previous investigations of Olitzki & Godinger (1963) on *in vivo*- and *in vitro*-grown *S. typhi* it was shown that normal liver extract was able to enhance markedly the lethal effect of these pathogenic bacteria, when injected intraperitoneally into mice. In the above-described experiments we could determine that sublethal minimal amounts of *S. typhi* suspended in saline did not immunize, while the same quantities, about 10^2–10^3 bacteria, injected together with normal liver extract produced an immunizing effect. Also in an experiment reported by Olitzki & Kaplan (1963) it was demonstrated that at a 4 hr. incubation-time liver extract behaved differently from other organ extracts. While the majority of the organ extracts acted as growth-promoting substances *in vitro* and enhanced the bactericidal serum action on strain Ty 2, the normal liver extract acted mainly as an inhibitor of the bactericidal action. It has to be mentioned that this 2-fold effect, the promotion of growth in absence of serum and complement and the enhancement of bactericidal serum action in their presence, is not limited only to the majority of organ extracts. Michael & Braun (1959) described similar effects exerted by broth, glucose and certain amino acids on the susceptibility of *Shigella dysenteriae* and *Escherichia coli* to the bactericidal action of normal human serum.

Several properties of organ extracts from infected animals have already been demonstrated by previous experiments, namely the accumulation of infection-promoting substances and precipitinogens by Olitzki & Godinger (1963), and of

inhibitors of the bactericidal serum action by Olitzki & Kaplan (1963). The experiments described in this paper revealed an additional activity of the infected organ extracts: their immunogenic action. As in the previous communication, no indication has been found that these immunogenic substances are toxic. Even the most concentrated preparations of the immunogenic substances obtained by ethanol precipitation proved to be non-toxic.

There remains still to be investigated the relationship between these immunogenic substances and the enhanced immunogenicity of the *in vivo*-grown bacteria. The production of great quantities of *in vitro*-grown bacteria in the peritoneal cavity of the guinea-pig and their immunogenicity make possible a continuation of this investigation on the above lines.

SUMMARY

1. *Salmonella typhi*, strain Ty 2, grown *in vivo* and employed as acetone-dried vaccine possessed a higher immunizing potency than the descendants of the same parent strain grown *in vitro* and employed as vaccine.

2. When 2×10^8 *in vitro*-grown bacteria were employed as challenge, the immunizing effects of both types of vaccine were more marked than after administration of 2×10^8 *in vivo*-grown bacteria as challenge.

3. The higher potency of the *in vivo*-grown vaccine was apparent in all experiments, whether the challenge strain was grown *in vivo* or *in vitro*.

4. Immunogenic substances were isolated from infected organs of mice and guinea-pigs, and an immunogenic substance from the peritoneal fluid of the infected guinea-pigs was concentrated by precipitation with ethanol.

REFERENCES

Michael, J. G. & Braun, W. (1959). Modification of bactericidal effects of human sera. *Proc. Soc. exp. Biol., N.Y.*, **102**, 486.

Olitzki, A. L. & Godinger, D. (1963). Comparative studies on *Salmonella typhi* grown *in vivo* and *in vitro*. I. Virulence, toxicity, production of infection-promoting substances and DPN-ase activity. *J. Hyg., Camb.*, **61**, 1.

Olitzki, A. L. & Kaplan, O. (1963). Comparative studies on *Salmonella typhi* grown *in vivo* and *in vitro*. II. The effect of extracts from normal and infected organs on the bactericidal serum action on strains grown *in vivo* and *in vitro*. *J. Hyg., Camb.* **61**,, 21.

J. Hyg., Camb. (1963), **61**, 365
Printed in Great Britain

Intradermal TAB immunization against enteric infections

By P. N. BARDHAN, H. N. DUTTA and P. KRISHNASWAMI
Armed Forces Medical College, Poona

(*Received* 21 *March* 1963)

INTRODUCTION

The value of TAB vaccine in the prevention of enteric fevers is established. Ordinarily the vaccine is given subcutaneously, though the intradermal route has been recommended by Tuft (1931), Perry (1937), Longfellow & Luippold (1940). However, Luippold (1944), following further work, suggested that subcutaneous inoculations gave satisfactory enough results; he, however, added that in elderly persons or in others where allergy was suspected, the intracutaneous method was to be preferred. Observations recently made in India include those by Karani, Jadeja & Ganguli (1957), Kalra, Ganguli & Bardhan (1960) and Karani, O'Leary & Luxa (1960). The problem has also been studied among British troops by Barr, Sayers & Stamm (1959). The present study is an attempt to evaluate the antibody response and the clinical reactions to TAB vaccine administered subcutaneously or intradermally, as a primary immunization procedure amongst Indian troops.

MATERIALS AND METHODS

The vaccine

(i) The intradermal vaccine was prepared by Central Research Institute, Kasauli. It was designated 'Special TAB Vaccine', containing 5000 million organims per ml. (typhoid-2500 millions; paratyphoid A and partyphoid B 1250 millions each).

(ii) The vaccine given subcutaneously was the ordinary TAB vaccine prepared by C. R. I. Kasauli and used by the Armed Forces in India. It contains 2000 million organisms per ml. (typhoid 1000 millions, paratyphoid A and paratyphoid B 500 millions each).

Both the vaccines were heat killed and preserved with phenol.

The strains used in both vaccines were:

(i) Armed Forces Medical College strain *Salmonella paratyphi* A, no. 1564/X/156/P, isolated at the Department of Pathology of the College from a case (this strain is fairly common in this locality).

(ii) *S. paratyphi* B, Findley.

(iii) *S. typhi* Vi, Pillay.

(iv) *S. typhi* (Rawlings).

Strains (ii), (iii) and (iv) are well known, while strain (i) occurs frequently in India.

There was no difference apart from the concentration between the special and the ordinary vaccines.

The following doses were adopted, the interval between the doses being 14 days:

Intradermal group (i.d.): 1st dose—0·1 ml. of special TAB vaccine (500 million organisms). 2nd dose—0·2 ml. of special TAB vaccine (1000 million organisms).

Subcutaneous group (s.c.): 1st dose—0·5 ml. of the ordinary vaccine (1000 million organisms). 2nd dose—1·0 ml. of the ordinary vaccine (2000 million organisms).

It will be seen that the number of organisms introduced subcutaneously is twice the number of organisms given intradermally. Volumes, of course, are less in the intradermal method.

Trial subjects

The trial was carried out on fresh recruits of an Army Engineer group. These young healthy recruits, of age-group 17–19 years, were drawn from various parts of the country, and all had good physical and nutritional standards. They had had no previous TAB inoculation. A group of volunteers numbering 257 was divided by a method of random sampling into two groups. The trial group of 128 persons was given the special TAB vaccine by the intradermal route and the control group of 129 persons the ordinary TAB vaccine by the subcutaneous route. The intradermal injections were all given on the forearm and the subcutaneous injections in the deltoid region.

Techniques

Before inoculation the recruits were bled to determine the basal antibody levels. Agglutinin levels against AO, BO, TO, Vi, AH, BH and TH were determined by Felix's method for 'O' agglutination (Cruickshank, 1960) and by Dreyer's method for 'H' agglutination (Cruickshank, 1960). The bacterial suspensions used in these tests were the standard products of the Department of Pathology, Armed Forces Medical College, Poona.

The investigation was divided into two parts, clinical and immunological.

Clinical studies

Local and general reactions were recorded under five headings at 24, 48 and 72 hr. after inoculation: (i) temperature over 99° F.; (ii) inability to work; (iii) severe local pain; (iv) severe headache; (v) redness and swelling of diameter over 10 cm.

Immunological studies

Serological investigations were carried out before inoculation to determine the basal titre, then at 6 weeks, between 21 and 22 weeks, and finally at 59–60 weeks after the first TAB inoculations.

RESULTS

Clinical studies

The data on clinical reactions are given in Table 1. As regards rise of temperature there was no significant difference between the two groups after the first inoculation, but after the second inoculation the intradermal group showed a greater

incidence. There was no significant difference between the two groups regarding inability to work. The incidence of severe local pain, severe headache, and redness and swelling was generally higher in the intradermal group.

Immunological studies

The results in Table 2 indicate that:

(*a*) The basal titre is generally of the same order in both groups, but in subsequent determinations the titre is more often higher in the intracutaneous group.

(*b*) A distinct antibody rise is noticed at 21–22 weeks and 59–60 weeks; however, in the subcutaneous group, the titre at 60 weeks marks a reduction from a

Table 1. *Clinical reactions with subcutaneous and intradermal* TAB *inoculation*

Reaction	Dose	Period of observation (hr.)	No. of subjects examined		No. of positive reactions		Percentage		Remark
			s.c.	i.d.	s.c.	i.d.	s.c.	i.d.	
Temp. 99° F. and over	1st	24	129	128	23	16	17·8	12·5	—
		48	129	128	3	5	2·3	3·9	—
		72	129	128	0	2	0·0	1·6	—
	2nd	24	125	123	10	17	8·0	13·8	—
		48	125	123	1	12	0·8	9·8	**
		72	125	123	0	1	0·0	0·8	—
Inability to work	1st	24	129	128	20	12	15·5	9·4	—
		48	129	128	1	2	0·8	1·6	—
		72	129	128	0	0	0·0	0·0	—
	2nd	24	125	123	19	22	15·2	17·9	—
		48	125	123	2	3	1·6	2·4	—
		72	125	123	0	0	0·0	0·0	—
Severe local pain	1st	24	129	128	85	101	65·9	78·9	*
		48	129	128	58	86	45·0	67·2	**
		72	129	128	16	34	12·4	26·6	**
	2nd	24	125	123	53	107	42·4	87·0	**
		48	125	123	44	50	35·2	40·6	—
		72	125	123	10	19	8·0	15·4	—
Severe headache	1st	24	129	128	45	46	34·9	35·9	—
		48	129	128	13	39	10·1	30·5	**
		72	129	128	1	11	0·8	8·6	**
	2nd	24	125	123	38	55	30·4	44·7	*
		48	125	123	15	23	12·0	18·7	—
		72	125	123	1	4	0·8	3·3	—
Redness and swelling of diameter over 10 cm.	1st	24	129	128	47	65	36·4	50·7	*
		48	129	128	64	105	49·6	82·0	**
		72	129	128	10	85	7·8	66·4	**
	2nd	24	125	123	22	77	17·6	62·6	**
		48	125	123	60	104	48·0	84·6	**
		72	125	123	25	56	20·0	45·5	**

s.c. = Subcutaneous. i.d. = Intradermal.

* Difference significant as tested by χ^2 ($P < 0{\cdot}05$).

** Difference significant as tested by χ^2 ($P < 0{\cdot}01$).

Table 2. *Agglutinin responses to* TAB *vaccine*

Titre	Basal s.c.	Basal i.d.	6 weeks s.c.	6 weeks i.d.	21–22 weeks s.c.	21–22 weeks i.d.	60 weeks s.c.	60 weeks i.d.
				(*a*) TO agglutinin				
—	12	22	0	1	2	0	4	2
10	22	15	4	1	0	0	0	0
20	49	38	43	21	4	2	6	2
40	25	33	42	33	7	5	10	4
80	20	20	30	25	26	11	22	5
160	1	0	2	23	34	23	21	11
320	0	0	0	8	24	33	17	27
640	0	0	0	2	11	29	11	24
1280	0	0	0	1	2	12	2	18
2560	0	0	0	0	0	1	0	0
Total	129	128	121	115	110	116	93	93
Geometric mean	21·6	19·9	36·3	65·3	149·0	297·8	120·9	333·9
Significance	—		$P < 0·01$		$P < 0·01$		$P < 0·01$	
No. with titre of 80 or more*	21	20	32	59	97	109	73	85
Percentage	16·3	15·6	26·4	51·3	88·2	94·0	78·5	91·4
				(*b*) AO agglutinin				
—	120	120	110	93	52	56	49	18
10	8	8	11	18	0	0	8	8
20	1	0	0	3	38	40	25	44
40	0	0	0	1	14	13	10	16
80	0	0	0	0	4	3	1	6
160	0	0	0	0	2	4	0	1
Total	129	128	121	115	110	116	93	93
Geometric mean	3·4	3·4	3·5	4·1	10·0	9·9	7·8	16·6
Significance	—		—		—		$P < 0·01$	
No. with titre of 20 or more*	1	0	0	4	58	60	36	67
Percentage	0·8	0·0	0·0	3·5	52·7	51·7	38·7	72·0
				(*c*) BO agglutinin				
—	101	97	45	31	17	20	19	12
10	24	25	38	38	0	0	6	5
20	4	5	35	38	24	21	28	25
40	0	1	3	7	38	28	22	22
80	0	0	0	1	14	22	14	17
160	0	0	0	0	13	17	4	11
320	0	0	0	0	4	8	0	1
Total	129	128	121	115	110	116	93	93
Geometric mean	4·1	4·3	8·2	10·2	32·2	36·7	20·8	30·4
Significance	—		—		—		$P < 0·01$	
No. with titre of 40 or more*	0	1	3	8	69	75	40	51
Percentage	0·0	0·8	2·5	7·0	62·7	64·7	43·0	54·8

Table 2 (*cont.*)

Titre	Basal s.c.	Basal i.d.	6 weeks s.c.	6 weeks i.d.	21–22 weeks s.c.	21–22 weeks i.d.	60 weeks s.c.	60 weeks i.d.
				(*d*) Vi agglutinin				
—	128	127	121	98	88	97	87	82
10	1	1	0	12	1	0	2	1
20	0	0	0	5	21	15	4	10
40	0	0	0	0	0	3	0	0
80	0	0	0	0	0	1	0	0
Total	129	128	121	115	110	116	93	93
Geometric mean	3·2	3·2	3·2	3·9	4·5	4·4	3·5	3·9
Significance	—		—		—		—	
No. with titre of 10 or more*	1	1	0	17	22	19	6	11
Percentage	0·8	0·8	0·0	14·8	20·0	16·4	6·5	11·8
				(*e*) TH agglutinin				
—	114	99	0	1	2	4	1	2
25	5	16	4	3	9	6	9	0
50	3	7	20	12	21	9	13	3
125	4	5	43	30	34	28	22	7
250	3	1	37	48	21	19	19	21
500	0	0	15	20	15	28	10	23
1250	0	0	2	1	4	16	13	27
2500	0	0	0	0	2	6	6	10
5000	0	0	0	0	2	0	0	0
Total	129	128	121	115	110	116	93	93
Geometric mean	6·8	8·1	155·3	183·6	146·9	240·7	203·5	502·5
Significance	—		—		$P < 0·01$		$P < 0·01$	
No. with titre of 50 or more*	10	13	117	111	99	106	83	91
Percentage	7·8	10·2	96·7	96·5	90·0	91·4	89·2	97·8
				(*f*) AH agglutinin				
—	119	101	1	2	1	7	7	2
25	3	18	6	8	5	8	18	1
50	2	4	22	25	30	11	11	4
125	2	2	33	33	32	26	14	12
250	3	3	49	34	18	26	15	24
500	0	0	6	13	20	24	20	28
1250	0	0	3	0	3	9	8	17
2500	0	0	1	0	1	4	0	5
5000	0	0	0	0	0	1	0	0
Total	129	128	121	115	110	116	93	93
Geometric mean	6·2	7·8	146·4	124·3	138·6	179·8	118·4	358·0
Significance	$P < 0·01$		—		$P < 0·05$		$P < 0·01$	
No. with titre of 50 or more*	7	9	114	105	104	101	68	90
Percentage	5·4	7·0	94·2	91·3	94·5	87·1	73·1	96·8

Table 2 (*cont.*)

	Basal		6 weeks		21–22 weeks		60 weeks	
Titre	s.c.	i.d.	s.c.	i.d.	s.c.	i.d.	s.c.	i.d.
			(*g*) BH agglutinin					
—	123	112	6	3	13	7	13	2
25	1	8	19	9	18	10	16	0
50	3	2	48	35	38	23	17	6
125	2	3	28	26	18	31	14	11
250	0	3	14	26	16	24	20	40
500	0	0	6	14	5	12	10	23
1250	0	0	0	2	2	7	3	9
2500	0	0	0	0	0	2	0	2
Total	129	128	121	115	110	116	93	93
Geometric mean	5·6	6·8	66·8	110·5	58·8	120·4	74·2	278·2
Significance	—		$P < 0·01$		$P < 0·01$		$P < 0·01$	
No. with titre of 50 or more*	5	8	96	103	79	99	64	91
Percentage	3·9	6·3	79·3	89·6	71·8	85·3	68·8	97·8

s.c. = Subcutaneous. i.d. = Intradermal.

* These or less are the titres ordinarily found in India among uninoculated population.

peak which had been reached earlier, in contrast with the intracutaneous group in which the titre at 60 weeks is generally higher than the earlier values.

It appears that the intradermal method of inoculation, with a lesser number of bacteria, has given better immunity, as judged by agglutination titres, than the routine subcutaneous method.

The results also indicate that the response to Vi antigen was low in all cases and that the response to AO antigen was generally lower than that to TO and BO antigens.

The high titres shown by an appreciable number of men at the basal level may mean that they had enteric fever or some other fever with common antigenic property sometime before the trial began.

DISCUSSION

In this study, clinical features such as raised temperature, pain and swelling and subjective symptoms like headache are more marked with the intradermal method. It was reported earlier by Karani *et al.* (1960) that the intradermal method produced less local and general reactions than the subcutaneous route; they gave 0·1 ml. (200 million organisms) to 115 Service personnel as an annual booster dose. The British trial by Barr *et al.* (1959), showed that the reaction pattern was milder with the intradermal route. Our observations, however, do not confirm those findings.

The results of agglutinin responses to the TAB vaccine over a year (Table 2) show that the antibody response by the intradermal route is generally higher and

perhaps lasts a little longer than that given by the subcutaneous route. This agrees with the findings of Tuft, Yagle & Rogers (1932) who noticed that the intradermal route produced a better and more lasting response than all the other routes except the intravenous, but by the intravenous route the titres were maintained for shorter periods. They used one-tenth of the dose; the number of organisms was not mentioned. These workers also reported that the antibody response was not associated with constitutional reactions, a finding which is not corroborated by the present study. This may be due to the fact that the number of organisms employed in the present trial by the intradermal route is more than that given by Tuft *et al.* (1932).

The British trial (Barr *et al.* 1959) employing 500 million organisms of TAB vaccine by the intradermal route and 1250 million organisms by the subcutaneous route showed approximately the same serological responses to the TAB components using either route. The trial of Kalra *et al.* (1960) also showed poor antibody rise with the intradermal route; the number of organisms introduced by these workers were 200 and 400 million organisms by the intradermal and 1000 and 2000 million organisms by the subcutaneous route, with an interval of one week between the two doses. Neither of these trials was of sufficient duration; the antibody responses were determined a fortnight after the last injections.

In the present study, it is noticed that agglutinin response to AO antigen is poor in both the groups as compared to those to TO and BO antigens. The reason for this poor antibody response to *Salm. paratyphi* A in the vaccine is not known. Whether increasing the number of this organism in the TAB vaccine will stimulate better antibody response, remains to be determined. This is important from the preventive aspect of immunization in India, where paratyphoid A is considered to be the most common enteric infection after typhoid.

SUMMARY

To compare the effects of subcutaneous and intradermal methods of administration of TAB vaccine for primary immunization against enteric infections an investigation was carried out on 257 Army recruits.

The general and local reactions were more unfavourable by the intradermal method. The agglutinin responses were better with the intradermal route.

Our thanks are due to Colonel J. V. P. Braganza, Commandant of an Engineer Group, for permitting the study to be undertaken in his unit; to Captain B. K. Bhattacharya, Medical Officer of the unit, for his help and technical co-operation; to Dr J. B. Shrivastava, Director, Central Research Institute, Kasauli, for kindly preparing the 'Special TAB Vaccine' for this trial; to Subedar S. B. Roy, Flight-Sergeant K. P. Sukumaran and Jemadar C. D. Dubey, for technical assistance, and to Director General, Armed Forces Medical Services for permission to publish.

REFERENCES

BARR, M., SAYERS, M. H. P. & STAMM, W. P. (1959). Intradermal T.A.B.T. vaccine for immunization against enteric. *Lancet*, i, 816.

CRUICKSHANK, R. (1960). *Mackie and McCartney's Hand-book of Bacteriology*, 10th ed., pp. 315–18. Edinburgh and London: E. and S. Livingstone Ltd.

KAILRA, S. L., GANGULI, S. & BARDHAN, P. N. (1960). T.A.B. vaccine and the Indian Armed Forces. *Armed Forces med. J. (India)*, **16**, 9.

KARANI, N. D. P., JADEJA, A. K. & GANGULI, S. (1957). Intracutaneous T.A.B. inoculation. *Armed Forces med. J. (India)*, **13**, 61.

KARANI, N. D. P., O'LEARY, F. D. & LUXA, S. J. (1960). Intracutaneous T.A.B. inoculation. *Armed Forces med. J. (India)*, **16**, 15.

LONGFELLOW, D. & LUIPPOLD, G. F. (1940). Typhoid vaccination studies—revaccination and duration of immunity. *Amer. J. publ. Hlth*, **30**, 1311.

LUIPPOLD, G. F. (1944). Typhoid vaccination and studies. IX. intracutaneous versus subcutaneous vaccination for initial immunization. *Amer. J. publ. Hlth.* **34**, 1151.

PERRY, R. M. (1937). Comparison of typhoid 'O' and 'H' agglutinin responses following intracutaneous and subcutaneous inoculation of typhoid, paratyphoid A and B vaccine. *Amer. J. Hyg.* **26**, 388.

TUFT, L. (1931). Active immunisation against typhoid fever with particular reference to an intradermal method. *J. Lab. clin. Med.* **16**, 552.

TUFT, L., YAGLE, E. M. & ROGERS, S. (1932). Comparative study of the antibody response after various methods of administration of mixed typhoid vaccine. *J. infect. Dis.* **50**, 98.

J. Hyg., Camb. (1963), **61**, 373
Printed in Great Britain

Some observations on the use of the Ouchterlony gel diffusion technique in the study of myxomatosis

By PAUL J. CHAPPLE
Ministry of Agriculture, Fisheries and Food, Infestation Control Laboratory, Worplesdon, Surrey

E. T. W. BOWEN
Microbiological Research Establishment, Porton, Wilts

AND N. D. LEWIS
Ministry of Agriculture, Fisheries and Food, Infestation Control Laboratory, Worplesdon, Surrey

(*Received* 26 *March* 1963)

INTRODUCTION

Several workers have studied the immunological response of the rabbit to myxoma and fibroma viruses. Mansi (1957) and Mansi & Thomas (1958) used the Ouchterlony gel diffusion precipitin technique to study the course of the immunological response. In the latter paper a suggestion was made that it might be possible to differentiate between typical and atypical forms of the disease myxomatosis by the use of the gel test. The present work is an extension of the Mansi & Thomas investigation. This work is in two parts. First, there is a study of laboratory infections of domestic rabbits and this was carried out at the National Institute for Medical Research (N.I.M.R.,) Mill Hill; the Microbiological Research Establishment (M.R.E.), Porton, and the Ministry of Agriculture, Fisheries and Food, Field Research Station, Worplesdon. The second part is concerned with the study of the antigen and antibody in wild rabbit carcasses obtained from one outbreak of the disease.

MATERIALS AND METHODS

The gel diffusion technique as reported by Darbyshire (1962) for the study of mucosal disease of cattle was closely followed. This technique is almost identical with that employed at the Central Veterinary Laboratory for the diagnosis of myxomatosis. The size and distances apart of the wells used in these experiments are shown in Fig. 1*a*.

Antigens

Primary lesion, lung and blood clot (or serum) were used as test material. The tissues were cut up so that they fitted cleanly into the wells prepared in the agar. The control (positive) antigen was lesion homogenate from an infected rabbit.

Antisera

The control (positive) antiserum was a serum pool from rabbits which had recovered from myxomatosis.

Control antiserum and antigen were samples of that used at the Central Veterinary Laboratory.

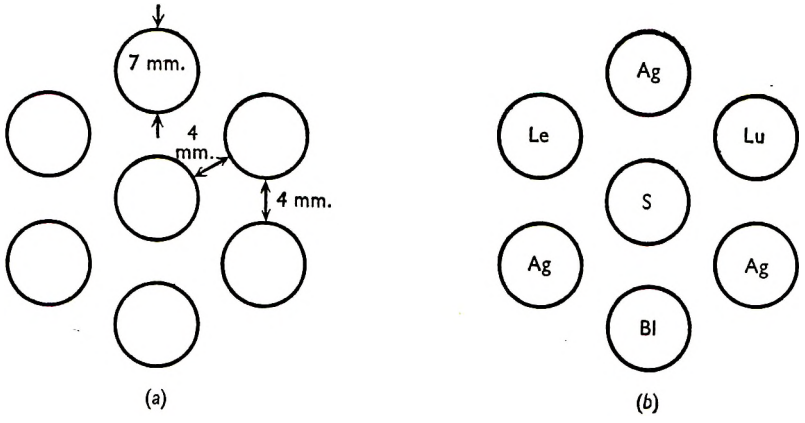


Fig. 1. (*a*) Sizes and distance of wells. (*b*) Arrangement of tissues and controls. Ag, known myxoma antigen (control); S, myxoma antiserum. From animals under test: Le, lesion material; Lu, lung; Bl, blood clot or serum.

Agar

Two agar media were used:

(1) Oxoid Ionagar no. 2 (1·5 %, w/v) in 0·85 % (w/v) sodium chloride buffered with 0·01 M phosphate and with 0·5 % (w/v) phenol to control growth of micro-organisms. The final pH was adjusted to 7·2, using N/10 sodium hydroxide. Plates were prepared by pouring 12 ml. agar medium in 9 cm. diameter Petri dishes. Used at N.I.M.R., Mill Hill.

(2) Oxoid Ionagar no. 2 (1·5 % w/v) in 0·01 M sodium barbitone/hydrochloric acid, buffer pH 7·2. 20 ml. agar was poured into 9 cm. diameter Petri dishes. Used throughout, except at N.I.M.R.

Rabbits

Domestic rabbits weighing 4–4½ lb. were kept in individual cages in rooms which had a constant temperature (Worplesdon, 15·6° C. (60° F.); M.R.E., 18·5° C. (65° F.); N.I.M.R., not recorded).

Wild rabbits were obtained from a large population occupying an area of approximately 200 acres of a Yorkshire dale. One hundred and twenty-four rabbit carcasses were obtained over a period of 3 days; the majority were shot but a few were taken by snaring and dogs. On receipt in the laboratory pieces of eyelid, lung and blood clot were removed from each carcass and stored at −20° C. until required. A record was kept of the weight and sex of each rabbit.

Virus strains. These are listed in Table 1.

Table 1. *Virus strains*

Name	Synonyms and isolation	Origin	No. of laboratory passages
A & H	Mouse brain adapted (Andrewes & Harisijades, 1955)	Mouse brain adapted	Several
Cornwall	England/Cornwall/4–54/1 (Hudson, 1954, unpublished; Rowe, Mansi & Hudson, 1956)	Diseased wild rabbit	Several
Glenfield	Aust./Dubbo/2–51/1 (see Fenner & Marshall, 1957)	Naturally infected wild rabbit	*c.* 100
Cornwall–Port Looe	TPM 6/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Atro (R244)	England/Sussex/10–54/1 (Andrewes, Muirhead-Thomson & Stevenson, 1956)	From mosquito pool (*Anopheles maculipennis atroparvus*)	Several
Brecon	MX 4298 (Chapple & Bowen, 1963)	Diseased wild rabbit	0
KM13	Aust./Corowa/12–52/2 (Myers, Marshall & Fenner, 1954)	From pool of *A. annulipes*	1
Cornwall–Tor Point	MX4333 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bere Ferrers	TPM 1/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bere Ferrers	TPM 2/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bideford	TPM 3/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Devon—Bideford	TMP 4/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Cornwall–Tor point	TPM 5/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
St Austins Dale (Yorks)	TPM 13/62 (Chapple & Lewis, 1962, unpublished	Diseased wild rabbit	0
St Austins Dale (Yorks)	TPM 15/62 (Chapple & Lewis, 1962, unpublished)	Diseased wild rabbit	0
Durham	MX4297 (Chapple & Bowen, 1963)	Diseased wild rabbit	0
Notts	England/Nottingham/4–55/1 (Hudson, Thompson & Mansi, 1955)	Diseased wild rabbit	?

EXPERIMENTAL PROCEDURE

(*a*) *Laboratory infections*

Rabbits which were considered to be susceptible to myxomatosis were injected intradermally in the shaved right flank, by means of a virus-contaminated needle. The number of rabbits used for each strain is indicated in Table 2. These animals were subsequently kept under observation and the infection was allowed to run its normal course with the exceptions as indicated in Table 2. At death, blood,

Table 2. *Summary of results using laboratory rabbits*

No. of rabbits examined	Virus strain	Virulence (grading according to method of Fenner & Marshall, 1957)	Mean survival time and range in days	Gel test result no. rabbits (with their survival time in days in parentheses)			
				Antigen only	Antigen and antibody	Antibody only	Recoveries
9	A & H	I	14 (10–21)	9 (10, 10,* 10,* 10,* 13, 14, 13, 14, 21)	0	0	0
5	Cornwall	I	1 failed to be infected 14 (13–15)	4 (13, 13, 15, 15)	0	0	0
15	Glenfield	I	12·6 (8–24)	12 (13, 13, 13, 14, 24, 11, 11, 11, 8, 9, 13)	3 (13, 13, 13	0	0
5	TPM 6/62	II	13·5 (10–18)	5 (10, 12, 14, 14, 18)	0	0	0
8	Atro (R244)	III	22 (16–25)	7 (25, 23, 22, 16, 23, 25, 20)		1 (recovery)	1
15	Brecon	III	24 (17–47)	6 (19, 28, 19, 17, 22, 21)	6 (25, 33, 26, 24, 47, 35)	3 (2 recoveries 85†)	3
5	Brecon	III	Killed at 6 days	3‡ (—)	0	0	0
5	KM 13	III	19·3 (16–22)	3 (16, 20, 22)	0	2 (recoveries)	2
5	MX 4333	III	18·4 (10–24)	2 (18, 13)	3 (21, 10, 24)	0	0
5	TPM 1/62	III	21·3 (16–25), 1 failed to be infected	1 (16)	2 (23, 25)	1 (recovery)	1
4	TPM 2/62	III	24 (22–25)	1 (22)	3 (25, 26, 23)	0	0
5	TPM 3/62	III	18·8 (16–27)	4 (16, 16, 17, 18)	1 (27)	0	0
5	TPM 4/62	III	18·2 (15–22)	4 (13, 18, 23, 15)	1 (22)	0	0
5	TPM 5/62	III	18·0 (14–20)	3 (20, 17, 19)	2 (20, 14)	0	0
5	TPM 13/62	III	16·6 (14–21)	3 (12, 21, 21)	2 (14, 15)	0	0
5	TPM 15/62	III	16·2 (11–22)	3 (11, 13, 15)	2 (22, 20)	0	0
5	Durham	IV	2 failed to be infected	0	0	3 (recoveries)	3
4	Notts	IV	2 at 17 days, 1 died at 17 days, 1 failed to be infected	3 (17,* 17,* 17)	0	0	0

* Killed.

† Rabbit considered recovered, death was due to a cause other than myxomatosis.

‡ Two showed no antibody or antigen.

lung and primary lesion material were removed and tested against control (positive) antigen and antiserum.

The control serum was used undiluted; the respective dishes were filled to capacity. The arrangement of tissues and controls was always as in Fig. 1*b*. The plates were either incubated at 22° C. or left on the bench at room temperature. The plates were examined daily for 3 days before the result was finally recorded. Reading was carried out in a darkened room using an intense oblique light source.

(*b*) *Natural infections*

Since the original epizootic of 1953–55 samples of myxomatous rabbits have been sent regularly to the Central Veterinary Laboratory for examination by the gel-diffusion technique. A rabbit carcass was sent from the outbreak now under discussion and material from this rabbit was sent to Worplesdon. Virus was extracted from the material and injected into a group of five rabbits to assess the virulence according to the method originally employed by Fenner & Marshall (1957) and later used in this country by Chapple & Bowen (1963). All the rabbits died within 21 days, giving a mean survival time of 16·6 (range 14–21) days. On the basis of this mean survival time, and the clinical symptoms, the disease was assessed as being moderately severe. It was impossible to distinguish the virus causing this outbreak from that which produced the original epizootic in 1953–55 simply on the basis of the appearance of the rabbits in the field. The rabbit material used in the experiments now described was obtained about 10 weeks after the sample which was used to ascertain the mean survival time. An assessment of the virulence of the virus at this time resulted in a mean survival time of 19 (range 18–21) days.

All the tissues extracted from the rabbit carcasses were put up against known myxoma antigen and the pooled sera of recovered rabbits. On receipt of the rabbit carcasses a visual assessment of the clinical condition was made. The obvious absence of disease and obvious infection were labelled 'Clean' and 'Infected' respectively. The remainder were divided into two groups; first, those that looked reasonably healthy but owing to the condition of the carcass it was not possible to be certain; these were labelled '?Clean'. Secondly, those carcasses which were suspected of being infected but were also in such a condition as to make a positive diagnosis impossible, were designated '?Infected'. The clinical diagnosis is compared with the diagnosis using the gel test, in Table 3.

Figures 2–4 indicate in diagrammatic form some of the possible results in the gel test.

Fig. 2*a* shows the presence of antigen in all the tissues tested. Fig. 2*b* shows the presence of antibody in lung and blood clot; antigen is still present in the lesion material. It is possible to get antibody present in all the tissues tested. A negative reaction (no antibody or antigen detected) is shown by Fig. 4. These are the results which are frequently seen. However, there are occasions when only weak reactions are detected and two of these are illustrated in Figs. 3*a* and *b*. When an animal is killed early in the course of the disease (i.e. before generalization of the virus) only antigen is detectable and this is found exclusively in the site of inoculation.

Table 3. *Comparison of the clinical and gel test diagnosis and the attempted differentiation of virus by means of the gel test*

(Figures indicate numbers of rabbits.)

	Gel test diagnosis				
	Infected				
Clinical condition	Typical	Atypical	Total	Immune	Susceptible
Clean	4	10	14	1	20
Infected	23	55	78	1	1
? Infected	1	2	3	0	1
? Clean	0	1	1	0	2
Unknown	1	1	2	0	0
Total	29	69	98	2	24

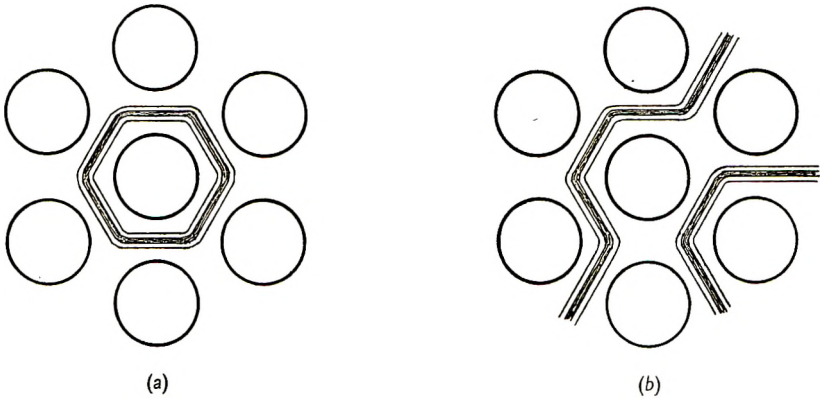


Fig. 2. (*a*) One of the possible gel results which would lead to a diagnosis of 'typical' (antigen only detected). (*b*) One of the possible gel results which would lead to a diagnosis of 'atypical' (antigen and antibody detected in the test materials).

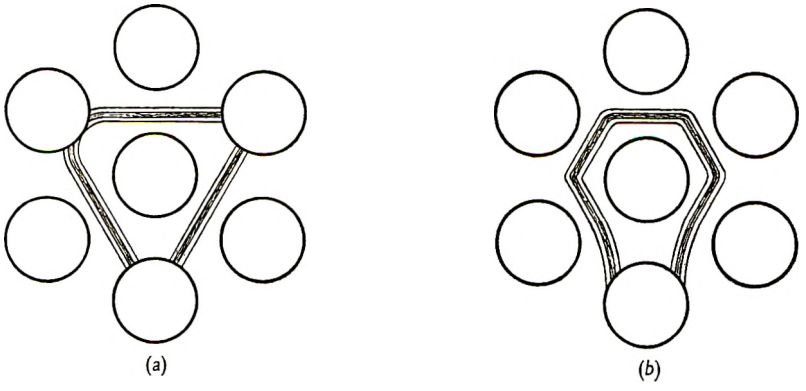


Fig. 3. (*a*) Weak 'antigen' reaction with lesion material. (*b*) Weak antibody reaction from blood.

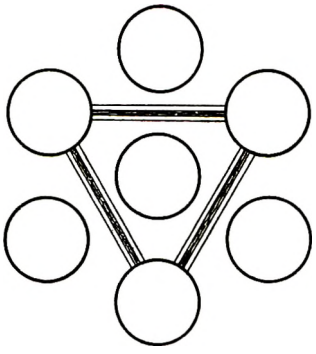


Fig. 4. Negative reaction (no antibody or antigen detected in the test materials).

RESULTS

The results of the gel tests in laboratory rabbits together with the mean survival times and the virulence classification of the virus strains are summarized in Table 2.

The wild-rabbit carcasses which were diagnosed as infected on the basis of the gel test results were typed according to the tentative hypothesis of Mansi & Thomas (1958). This hypothesis is summarized below.

Typical—only antigen detected in any or all of the material tested.

Atypical—any instance in which antibody is found with or without the presence of antigens except where antibody is detected only in the blood.

Immune—antibody detected only in the blood.

Susceptible—no antigen or antibody detected.

The effect of applying this to the results obtained with the sample of rabbits now under discussion is also shown in Table 3.

DISCUSSION

Mansi (1957) and Mansi & Thomas (1958) showed that myxoma and fibroma viruses could be identified by means of a modified Ouchterlony test in agar gel. Subsequently this test has been used for the routine diagnosis of myxomatosis at the Central Veterinary Laboratory, Weybridge. On the basis of the pattern produced by the precipitation lines with certain strains of myxoma virus Mansi & Thomas (1958) suggested, in their discussion, that it might be possible to differentiate between what they referred to as 'typical' and 'atypical' forms of the disease. Unfortunately the terms typical and atypical have been used, by some people, as if they were synonymous with the disease caused by virulent and attenuated forms of virus respectively although nothing has been published which would lead to such a conclusion.

However, it should be remembered that the hypothesis stems mainly from the work reported by Mansi & Thomas (1958) with two field strains of virus; the fully virulent 'Cornwall' (Rowe, Mansi & Hudson, 1956) and the supposedly attenuated 'Nottingham' strain (Hudson & Mansi, 1955; Fenner & Marshall, 1955). These

two strains were taken as representative of those causing 'typical' and 'atypical' infections respectively. The latter strain (Nottingham) has been shown to be a mixture of two strains—one fully virulent and the other a markedly attenuated strain (Fenner & Marshall, 1957).

In the present work it was thought advisable to examine several strains of each major type (fully virulent and attenuated). The relevant experimental results are summarized in Table 2.

As a working hypothesis it was assumed that all fully virulent strains of virus would yield the same result and that all attenuated strains would yield the same result. The experiments recorded in this paper indicate that this was too broad a generalization. Out of fifteen rabbits infected with the Glenfield strain of virus (a fully virulent Australian strain used for initiating epizootics) nine behaved in the expected manner. However, three rabbits gave a picture which would have definitely resulted in a report of atypical being given, i.e. antibody was detected as well as antigen. It is significant that these three rabbits were not ones which had a longer than normal survival time as one might have expected if the Mansi & Thomas hypothesis was correct. On the contrary one rabbit which was classified as 'typical' in fact survived for 24 days which is certainly unusual for this strain (F. Fenner, personal communication). The fact that antibody is not detected in the gel test does not necessarily mean that the strain of virus concerned is 'typical' fully virulent. An examination of Table 2 shows that of the rabbits inoculated with attenuated strains of virus, seventy-five died or recovered. There were ten recoveries (including the rabbit which died at 85 days) and of the remainder twenty-two showed antibody and antigen giving a gel diagnosis of atypical, and forty-three showed antigen only, giving a gel diagnosis of typical. However, because the strains of virus which caused the disease were known to be attenuated it would seem that the postulate of Mansi & Thomas (1958) does not always hold good and that it appears on the basis of these laboratory infection experiments that interpretation of the results of gel diffusion tests must be made with great caution.

Because of the suggestion that there might be significantly longer mean survival times obtained from wild rabbits, when compared with the domestic form (Fenner & Marshall, 1957) it was considered necessary to assess the gel test using a large population of wild rabbits. It was not possible to obtain a large number of live wild rabbits for use in the laboratory, nor to predict effects on the course of the disease of keeping live wild rabbits in captivity. Therefore, the rabbits studied were obtained from a large natural population which had had myxomatosis present for about 10 weeks. The sample was obtained over a period of 3 days. It is not suggested that the sample obtained (124 rabbit carcasses) was random but it was considered sufficiently large to include a number of normal and possibly recovered rabbits.

The first point to be examined was whether the gel test could provide information purely on the presence or absence of disease, over and above the information obtained by inspection of a carcass. The second point was whether the tentative suggestion of Mansi & Thomas (1958), that the gel test could differentiate between typical and atypical forms of disease, was tenable in a wild rabbit population.

An examination of Table 3 shows that of eighty rabbits which were obviously infected, seventy-eight gave a positive gel test confirming infection. In one of the remaining two, antibody only was detected (in the blood) giving a gel diagnosis of recovered. The second carcass did not show antibody or antigen in the materials tested. It is known that rabbits in the early stages of recovery often show marked nodular lesions and these would lead to a clinical diagnosis of infected. Conversely, it is possible, very occasionally, to pick pieces of tissue from a carcass which do not contain antigen or antibody in demonstrable amounts and this could explain the infected carcass which was typed as susceptible. These results are quite satisfactory but when one examines those obtained from rabbits which were called clean (after clinical inspection) then the situation is not satisfactory. Thirty-five rabbits were described as clean and of these fourteen were found to be infected, one was typed as recovered and twenty were typed as susceptible as a result of using the gel test. However, a closer examination of the fourteen rabbits typed as infected shows that only five had detectable antigen and the remaining nine had antibody only. From this it would be safe to assume that these nine rabbits were recovering or had recovered from the disease.

In the remaining three groups, ?Infected, ?Clean and Unknown, there are insufficient numbers to reach any definite conclusion. However there is the suggestion that the provisional clinical diagnoses were justified.

Table 4 is similar to the top line of Table 3, but makes use of a revised definition of a recovered (≡ immune—Mansi & Thomas, 1958) rabbit. It is suggested that this is a rabbit which is clinically clean and shows antibody alone in any or all of the tissues examined. Using this revised definition only five rabbits were classified as infected; of these, four showed antigen only, thus suggesting that they were in the early stages of the disease.

Table 4. *Comparison of clinical and gel test diagnosis using the revised definition of a recovered rabbit*

Clinical diagnosis	Gel test diagnosis			
	Infected	Recovered	Susceptible	Total
Clean (1)	14	1	20	35
Clean (2)	5	10	20	35

Clean (1): gel test interpretation as in Table 3. Clean (2): gel test interpretation revised.

The outbreak of myxomatosis under discussion was caused by a single virus introduction and we have reason to believe that no further introductions were made. The results are summarized in Table 3. The definitions for the gel test categories are those which are in current use at the Central Veterinary Laboratory and which have been described previously. From these results it would appear that there are two virus strains present, a fully virulent and an attenuated strain. As a result of our knowledge of the history of the outbreak, together with laboratory assessment of virulence, we have concluded that there was only one virus

strain involved. Therefore, the apparent differentiation shown by the gel test results was not valid. The course of the outbreak of the disease was much slower than that associated with disease caused by fully virulent virus. This slowness might have been caused by lack of suitable vectors. However, flea counts on rabbits sent in to the laboratory since the start of the outbreak suggest that there are adequate numbers of vectors. Of twenty rabbits examined we found from three to thirty-six rabbit fleas (*Spilopsyllus cuniculi*) present on each rabbit and the average was nineteen. Mead-Briggs (1963) has shown that the rabbit flea is a more mobile vector than was hitherto supposed. Thus any slowness in the spread of the disease within the population was probably due to the nature of the virus rather than the vector.

We have concluded that the gel test can provide valuable information on the infection and the recovery rate of wild rabbit populations provided that the clinical diagnosis is taken into consideration. However, the results show that it is impossible to differentiate fully virulent from attenuated virus using this technique.

SUMMARY

1. Laboratory rabbits were infected with seventeen different preparations of myxoma virus (this probably represents thirteen strains of which four could be classified as fully virulent and the rest as attenuated, two being markedly so).

2. One hundred and twenty-four wild rabbit carcasses were obtained from a rabbit infestation in the throes of an outbreak of myxomatosis.

3. All the carcasses were examined for the presence or absence of myxoma antigens and antibodies by a modified Ouchterlony gel-diffusion precipitin test.

4. As a result of (3), we have concluded that the gel test can provide confirmation of the presence or absence of myxomatosis. It is particularly useful in cases of doubt about the clinical diagnosis. The test can also give a reasonable indication of the number of recovered rabbits in a population and, more important still, it can give an indication of the recovery rate during an outbreak of disease. However, attempts to differentiate between fully virulent and attenuated strains of virus were unsuccessful.

We are extremely grateful to the Directors of the National Institute for Medical Research, Mill Hill, and the Microbiological Research Establishment, Porton, for the facilities made available whilst the laboratory was being prepared at Worplesdon. To Sir Christopher Andrewes, Dr J. C. N. Westwood and Prof. Frank Fenner we extend our thanks for help and encouragement. A special word of appreciation is due to Mr R. A. R. Matthews and other members of the Ministry of Agriculture's pests staff who organized the collection of the wild rabbits.

REFERENCES

ANDREWES, C. H. & HARISIJADES, S. (1955). The propagation of myxoma virus in one day old mice. *Brit. J. exp. Path.* **36**, 18.

ANDREWES, C. H., MUIRHEAD-THOMSON, R. C. & STEVENSON, J. P. (1956). Laboratory studies of *Anopheles atroparvus* in relation to myxomatosis. *J. Hyg., Camb.*, **54**, 478.

CHAPPLE, P. J. & BOWEN, E. T. W. (1963). A note on two attenuated strains of myxoma virus isolated in Great Britain. *J. Hyg., Camb.*, **61**, 161.

DARBYSHIRE, J. H. (1962). Agar gel diffusion studies with a mucosal disease of cattle. I. Preliminary results with the technique. *Res. vet. Sci.* **3**, 118.

FENNER, F. & MARSHALL, I. D. (1955). Occurrence of attenuated strains of myxoma virus in Europe. *Nature, Lond.*, **176**, 782.

FENNER, F. & MARSHALL, I. D. (1957). A comparison of the virulence for European rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in the field in Australia, Europe and America. *J. Hyg., Camb.*, **55**, 149.

HUDSON, J. R. & MANSI, W. (1955). Attenuated strains of myxomatosis virus in England. *Vet. Rec.* **67**, 746.

HUDSON, J. R., THOMPSON, H. V. & MANSI, W. (1955). Myxoma virus in Britain. *Nature, Lond.*, **176**, 783.

MANSI, W. (1957). The study of some viruses by the plate gel diffusion precipitin test. *J. comp. Path.* **67**, 297.

MANSI, W. & THOMAS, V. (1958). Serological investigation in the study of myxoma and fibroma viruses. II. The gel diffusion precipitin test. *J. comp. Path.* **68**, 188.

MEAD-BRIGGS, A. R. (1963). Some experiments concerning the interchange of rabbit fleas, *Spilopsyllus cuniculi* (Dale), between living rabbit hosts. (To be published.)

MYERS, K., MARSHALL, I. D. & FENNER, F. (1954). Studies in the epidemiology of infectious myxomatosis of rabbits. III. Observations on two succeeding epizootics in Australian wild rabbits on the Riverina plain of south-eastern Australia, 1951–1953. *J. Hyg., Camb.*, **52**, 337.

ROWE, B., MANSI, W. & HUDSON, J. R. (1956). The use of fibroma virus (Shope) for the protection of rabbits against myxomatosis. *J. comp. Path.* **66**, 290.