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CORRIGENDUM

W. Plowright and B. McCulloch, *J. Hyg., Camb.* (1967). **65**, 343–58

On page 344, paragraph 2, line 3, *for* $10^{6\cdot2}$ *read* $10^{0\cdot2}$

J. Hyg., Camb. (1967), **65**, 435
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The effect of the addition of *Bordetella parapertussis* suspensions on the protective power of the pertussis component of diphtheria–tetanus–pertussis vaccines

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(*Received* 10 *January* 1967)

INTRODUCTION

Previous experiments (Köhler-Kubelka, 1962) have shown that the addition of a parapertussis component to diphtheria–tetanus–pertussis vaccine lowered to some extent the agglutinogenic potency of the pertussis component. In the present work we have continued our investigations into this problem in order to find out to what extent the addition of the parapertussis organisms reduces the agglutinogenic potency of the pertussis component, and to find out whether the addition affects the protective power of pertussis vaccines as measured by active immunization tests in mice.

METHODS

Three batches of diphtheria–tetanus–pertussis (DTP) vaccine, numbers 26, 27 and 28, were prepared containing in each 0·5 ml. (human) dose: 20 Lf diphtheria toxoid, 10 B.U. tetanus toxoid, 12×10^9 organisms of *Bordetella pertussis*, 2 mg. $AlPO_4$ and merthiolate to a final concentration of 1/10,000. Six samples of vaccine were prepared from each of the three batches and to five of them parapertussis suspension was added to give final concentrations per 0·5 ml. of $0{\cdot}25 \times 10^9$, $0{\cdot}5 \times 10^9$, 1×10^9, 2×10^9, and 3×10^9. The sixth sample of each batch was left free from parapertussis component.

The parapertussis component was prepared by mixing equal parts of *Bordetella parapertussis* strains no. 37/56, 16/58, 32/59 and 50/59. The vaccine was heated at 56° C. for 30 min. and merthiolate to a final concentration of 1/10,000 was added.

The 18 vaccine samples under test were tested for agglutinin production by the method of Evans & Perkins (1953). Batches of 15 female mice, each weighing 14 g., were inoculated with 0·5 ml. of each of the 18 samples and bled 24 days later. The agglutinin titres of each mouse for pertussis and for parapertussis were determined by the author's own modification (Köhler-Kubelka, 1957) of Detlor's (1951) micro-agglutination test. The results are expressed in Table 1 as the geometric mean titre produced in each group of mice.

The 18 vaccine samples were also tested by the active mouse protection test according to the Requirements for Pertussis Vaccine (1964). The ED 50 values (the calculated amount of vaccine that will protect 50 % of the mice) of all samples were calculated by the probit method and a comparison made between the test and the control vaccine samples.

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RESULTS

The results are presented in Table 1.

The average agglutinin titres for parapertussis ranged from 1/932 to 1/20,945. The mice that received the higher doses of parapertussis gave the higher agglutinin titres against parapertussis and vice versa. The mice that received the control vaccines without the parapertussis component had no agglutinin titres to parapertussis.

Table 1. *Agglutinin titres and protective properties of various pertussis–parapertussis components of DTP vaccines*

Vaccine batch no.	Pertussis (millions per mouse dose)*	Parapertussis (millions per mouse dose)	Agglutinin titre†		ED 50‡ (millions)
			Pertussis	Parapertussis	
26-1	2,000	500	171	11,578	57·68
26-2	2,000	333	106	10,240	74·28
26-3	2,000	166	335	2,793	50·08
26-4	2,000	83	673	2,360	30·44
26-5	2,000	41	683	932	48·88
26-6	2,000	0	705	0	48·16
27-1	2,000	500	256	19,588	89·56
27-2	2,000	333	132	20,940	121·92
27-3	2,000	166	150	5,184	94·68
27-4	2,000	83	250	4,456	94·16
27-5	2,000	41	336	1,750	102·04
27-6	2,000	0	414	0	81·28
28-1	2,000	500	34	19,456	332·16
28-2	2,000	333	100	20,945	197·24
28-3	2,000	166	150	7,240	311·48
28-4	2,000	83	140	5,497	131·08
28-5	2,000	41	120	2,078	237·08
28-6	2,000	0	198	0	102·20

* Composition of pertussis–parapertussis component: mouse dose one-sixth of human dose.

† Reciprocal of average agglutinin titre in each batch of mice.

‡ 26-1–26-6: $\chi^2 h = 3{\cdot}5671 < \chi^2 hk$; $\chi^2 p = 2{\cdot}5214 < \chi^2 hp$. 27-1–27-6: $\chi^2 h = 6{\cdot}2093 < \chi^2 hk$; $\chi^2 p = 9{\cdot}4795 < \chi^2 hp$.
28-1–28-6: $\chi^2 h = 2{\cdot}0124 < \chi^2 hk$; $\chi^2 p = 3{\cdot}2807 < \chi^2 hp$

The average agglutinin titres for pertussis were lower in the mice inoculated with the vaccine containing parapertussis; in general one can say that the fall in pertussis titre was inversely proportional to the rise in parapertussis vaccine dose. The agglutinin titres against parapertussis were several times greater than those against pertussis.

The protective power of pertussis vaccine in mice was expressed in ED 50. The results show no significant difference in the degree of protection conferred by the vaccines with or without the parapertussis component, nor was there any significant difference between the various parapertussis-containing vaccines whether they contained 2 % or 25 % of the pertussis component. A simple statistical analysis of

the samples showed that all of them satisfy the conditions of homogeneity and parallelism; the deviations from parallelism are not significant nor are the fluctuations in heterogeneity.

DISCUSSION

It can be seen from the results that the addition of a parapertussis component to diphtheria–tetanus–pertussis vaccine reduces the agglutinin titres to the pertussis component, this being particularly evident when the amount of the parapertussis added was 25 % of the pertussis component; with smaller amounts of parapertussis this difference is less striking.

The agglutinin-eliciting property of parapertussis organisms in mice is markedly higher than that of pertussis organisms, as has been reported previously by the author (Köhler-Kubelka, 1962) and was confirmed by Malivanova (1966). In a combined pertussis–parapertussis vaccine the substantially higher agglutinogenic potency of the parapertussis organisms seems to block the immunogenic apparatus of the mice and make it incapable of reacting adequately in producing agglutinin against the pertussis component. This observation led us to investigate whether this inhibitory influence on agglutinogenic potency was also reflected in the protective powers of pertussis vaccines.

As a rule, *Bordetella pertussis* causes a more severe illness of longer duration in children than does *B. parapertussis*, and the main purpose of vaccination is to fight successfully against *B. pertussis* infection. A possible inhibition of the protective power of pertussis component, caused by the addition of parapertussis organisms, would throw doubt on the efficacy of a combined diphtheria–tetanus–pertussis–parapertussis vaccine or would bring about the necessity of increasing the number of pertussis organisms or reducing the number of parapertussis ones. The results of these tests show that no significant reduction in the level of protection conferred by the pertussis component was found when the added parapertussis component was between 2 and 25 % of the pertussis. Our routine vaccine (DTPP) contains in a 0·5 ml. dose 12×10^9 pertussis organisms, 1×10^9 parapertussis organisms, with diphtheria and tetanus toxoids; this combination (26–3, 27–3, 28–3) produced in mice, we considered, a sufficiently high agglutinin titre for parapertussis while the mouse protective power of the pertussis component remained unchanged within the accuracy of the mouse assay.

SUMMARY

The addition of a parapertussis component to diphtheria–tetanus–pertussis vaccine reduces the agglutinogenic potency of the pertussis component in mice. The loss is evident when the amount of the parapertussis component added is 25 % of that of the pertussis component, while the influence of relatively lower amounts of parapertussis organisms is less striking.

The parapertussis component in these vaccines, amounting to 2–25 % of the pertussis component, does not affect the protective power of the pertussis component in mice.

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Staphylococcus aureus strains of phage-group IV

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(*Received* 23 *March* 1967)

Phage-group IV was established to include strains of *Staphylococcus aureus* which are lysed only by phage 42D (Report, 1953). This phage had been obtained by Wilson & Atkinson by adaptation of phage 42C (see Williams & Rippon, 1952). Macdonald (1946) found that a large proportion of staphylococci isolated from cows' milk in Britain were lysed only by this phage, and many subsequent authors have confirmed this observation. Strains from human sources are rarely lysed by this phage. Williams Smith (1948*a*,*b*) obtained six other phages from bovine staphylococci which appeared to have a range of specificity rather similar to that of phage 42D. With these phages, he obtained a number of pattern reactions with staphylococci lysed by phage 42D, and was also able to increase the proportion of typable strains among bovine staphylococci. It is now customary to refer to phages which resemble phage 42D in host-range as members of group IV, and to classify *Staph. aureus* cultures lysed only by these phages as group IV strains.

Phage 42D is the only group IV phage included in the international basic set of staphylococcal phages. Therefore, group IV strains are not typed with this set, but only grouped. Investigators of human staphylococcal disease have been quite satisfied with this situation, but the growing interest in animal staphylococci, particularly those of bovine origin, has created new problems. According to our own observations (Meyer, 1965) bovine *Staph. aureus* strains give reactions with the international basic set of typing phages nearly as often as do strains of human origin, but this is not necessarily identical with typing. Pulverer (1965) reported that he was able to differentiate only about 50 % of bovine staphylococci with the international basic set. As will be demonstrated later, this was owing to the inadequate recognition and differentiation of members of group IV.

Williams Smith (1948*a*, *b*) did not recommend the use of his additional phages for typing bovine staphylococci, because he thought that the differences in pattern he observed were caused mainly by resistance acquired through lysogenization of the strains with the respective phages. This pessimistic conclusion was not confirmed by the observations of Nakagawa (1960*a*). This worker (Nakagawa, 1960*b*) selected a number of new phages from *Staph. aureus* strains obtained from bovine milk. With the help of these phages he could subdivide bovine staphylococci which had been allotted to phage group IV with the international set of phages into two types within which a number of lytic patterns could be distinguished. Davidson (1961) also proposed the use of a number of new phages, in addition to some of the

phages of the international set, for the differentiation of bovine strains. Amongst them, phages 102, 107, 108 and 111 were members of group IV.

We will now discuss how the application of additional phages of group IV influences the classification of *Staph. aureus* strains from various sources.

MATERIALS AND METHODS

First we examined the results we had obtained by applying the phages of the international basic set (Blair & Williams, 1961; Report, 1963) to 8202 *Staph. aureus* strains in the years 1956–64, in order to find out what proportion of them were lysed by phage 42D either alone or in combination with other phages. This material was composed of: 2523 strains from human clinical material (pus, various secretions); 4308 strains from nasal swabs and other environmental investigations of human beings; 626 strains from foodstuffs; 745 strains from various animals (616 from cattle and 129 from other animals).

Table 1. *Phages used for investigation*

1. International basic set:*	
Gr. I	29, 52, 52A, 79, 80
Gr. II	3A, 3B, 3C, 55, 71
Gr. III	6, 7, 42E, 47, 53, 54, 75, 77, 83A
Gr. IV	42D
Gr. M ('Miscellaneous')	81, 187
2. Davidson's phages:	
Gr. I/III	101, 110
Gr. IV	102, 107, 108, 111
Gr. M. ('Miscellaneous')	115
3. Additional phages:	
Gr. III	31B
Gr. IV	42F
Gr. M. ('Miscellaneous')	44A, 78

* As constituted in 1962 (Report, 1963). Certain modifications have since been made at the 1966 meeting of the International Subcommittee on Phage-Typing of Staphylococci (Report, 1967).

We then tested 921 strains with a combined set of phages which will be described later. These included 307 isolated from bovine milk. All of them were coagulase positive when tested with human plasma, but with the aid of the bovine-plasma test (Meyer, 1966*a*, *b*) they were subdivided into 250 bovine-plasma coagulase positive and 57 bovine-plasma coagulase-negative strains. The remaining 614 strains were of human origin; 156 were isolated from nose-swabs of dairy workers, milkers and butchers, and 458 from clinical material. All were coagulase-positive with human plasma and none was positive with bovine plasma. Because there was no difference between the two groups in their reaction with phage 42D they were jointly evaluated.

The combined set of phages consisted of the international basic set (Report, 1963), together with seven of Davidson's phages (Davidson, 1961) and four others

(see Table 1). All strains were examined with this set at routine test dilution (R.T.D.). Those that were untypable at R.T.D. were tested with the same phages at R.T.D. × 1000.

RESULTS

We observed that the phages of group IV very frequently reacted together with phages of other groups, especially with phages of group III. In our epidemiological analysis we were unable to find a clear line of demarcation between strains that were lysed only by group IV phages and those which were also lysed by phages of other groups, to which we shall refer subsequently as 'phage-group IV/mixed', and we shall not make a fundamental distinction between them.

Table 2. *Reactions with phage 42 D, and with other members of the international basic set of phages, of 8202 strains of* Staphylococcus aureus

Origin of strains	No of strains examined	No. lysed by: Phage 42 D (alone or with other phages)	Phage 42 D alone	Phage 42 D at R.T.D.
Man	6831	104 = 1·5 %	23	19
Cow	616	214 = 34·7 %	111	93
Pig	51	6	1	4
Dog	32	0	0	0
Sheep	26	3 } 12 = 9·3 %	3	3 } 7
Fowl	18 } 129	2	1 } 5	0
Goat	1	1	0	0
Chinchilla	1	0	0	0
Foodstuff	626	41 = 6·5 %	14	17
Sum	8202	371	153	136

R.T.D. = routine test dilution.

Table 2 contains data concerning the frequency of reactions with phage 42D in *Staph. aureus* strains tested only with the basic set of phages. While this phage relatively seldom lysed strains of human origin (1·5 %), it lysed 30·3 % of strains from animal sources. Among the animal strains, however, it reacted with 34·7 % of bovine strains but only with 9·3 % from other animals. Only 23 of 104 human strains lysed by phage 42D were lysed only by this phage (22·1 %), but 111 of 214 bovine strains (51·9 %) were lysed exclusively by phage 42D. A larger proportion of the bovine strains (43·4 %) than of the human strains (18·2 %) which were lysed by phage 42D gave this reaction at R.T.D. Among the bovine strains, reactions with phage 42D occurred more often at R.T.D. among those lysed exclusively by phage 42D (72 of 111: 64·9 %) than among those belonging to phage-group IV/mixed (20 of 103: 19·4 %).

In Tables 3–5, the distribution of 921 strains of *Staph. aureus* among the various phage-groups as a result of typing with the international basic set, and with the combined set of phages, is contrasted. The difference was most impressive in the bovine-plasma coagulase-positive *Staph. aureus* strains from cows' milk (Table 3)

but is also perceptible in the bovine-plasma coagulase-negative bovine staphylococci (Table 4) and in the strains of human origin (Table 5).

The use of the additional group IV phages in the combined set increased the number of group IV strains among 250 bovine-plasma coagulase-positive *Staph. aureus* cultures from 37 to 170, and group IV plus group IV/mixed from 72 to 244 (Table 3). In other words, 97·6 % of the cultures were lysed by one or more of the group IV phages in the combined set, either alone or together with lysis by phages

Table 3. *Grouping of the phage-patterns of* 250 *bovine-plasma coagulase-positive strains of* Staphylococcus aureus *when tested with the basic set and with the combined set of phages*

	With basic set			With combined set			
Phage-groups	R.T.D.	R.T.D. × 1000	Total	R.T.D.	R.T.D. × 1000	Total	Difference
I: 80/81 + other phages	0	0	0	0	0	0	−5
I: other phages	3	4	7	2	0	2	
II	1	0	1	0	0	0	−1
III	20	62	82	1	0	1	−81
IV	29	8	37	168	2	170	+133
I/III	6	61	67	0	1	1	−83
II/III	2	5	7	0	0	0	
I/II/III	0	10	10	0	0	0	
I/IV	0	1	1	1	1	2	+39
II/IV	0	0	0	1	0	1	
III/IV	1	14	15	15	0	15	
I/III/IV	3	12	15	49	3	52	
II/III/IV	0	1	1	0	1	1	
I/II/III/IV	0	3	3	2	1	3	
Untypable			4			2	−2

Table 4. *Grouping of the phage-patterns of* 57 *bovine-plasma coagulase-negative strains of* Staphylococcus aureus *when tested with the basic set and with the combined set of phages*

	With basic set			With combined set			
Phage-groups	R.T.D.	R.T. × 1000	Total	R.T.D.	R.T.D. × 1000	Total	Difference
I: 80/81	1	0	1	1	0	1	−4
I: 80/81 + other phages	4	1	5	3	1	4	
I: other phages	5	2	7	3	1	4	
II	6	3	9	5	3	8	−1
III	8	3	11	7	3	10	−1
IV	0	0	0	1	0	1	+1
187	2	1	3	2	1	3	—
I/III	8	6	14	4	4	8	−6
I/IV	0	0	0	1	0	1	+5
III/IV	1	0	1	3	2	5	
I/III/IV	0	0	0	6	3	9	
II/III/IV	0	0	0	1	0	1	
Untypable	—		6	—	—	2	−4

Table 5. *Grouping of the phage-patterns of* 614 Staphylococcus aureus *strains of human origin when tested with the basic set and with the combined set of phages*

Phage groups	With basic set			With combined set			Difference
	R.T.D.	R.T.D. × 1000	Total	R.T.D.	R.T.D. × 1000	Total	
I: 80/81	112	20	132	112	17	129	−22
I: 80/81 + other phages	31	10	41	30	9	39	
I: other phages	49	45	94	45	32	77	
II	27	11	38	27	11	38	—
III	75	30	105	67	20	87	−18
IV	0	0	0	6	1	7	+7
187	9	44	53	9	44	53	—
I/II	0	1	1	0	1	1	
I/III	29	46	75	20	36	56	−19
I/II/III	1	1	2	1	1	2	
I/IV	0	2	2	6	9	15	+58
III/IV	0	2	2	10	13	23	
I/III/IV	2	6	8	11	21	32	
I/II/III/IV	0	1	1	0	1	1	
Untypable	—	—	60	—	—	54	−6

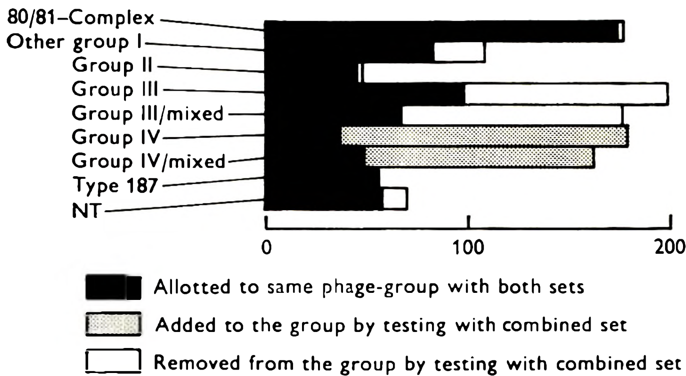

Fig. 1. Change in frequency of phage-groups when *Staphylococcus aureus* strains were tested first with the international basic set, and then with the combined set of phages.

of other groups. With the 57 bovine-plasma coagulase-negative strains from cows' milk (Table 4), there was little increase in the number of strains lysed only by the group IV phages, but the use of the combined set increased the number of group IV/mixed strains from 1 to 16 (1·8 % to 28·1 %). Similar results were obtained with the 614 human strains (Table 5), where the increase of group IV strains was from 0 to 7, and of group IV/mixed strains was from 13 to 71 (2·1 % to 11·6 %). It will be seen from Fig. 1 that the increase of group IV and group IV/mixed strains was almost exclusively at the expense of strains classified as group III and group III/mixed by the international basic set.

The results of typing at R.T.D. and at R.T.D. × 1000 with the two sets of phages may also be compared in Tables 3–5. With the basic set, a considerable minority

(8 out of 37) of the strains lysed only by phage 42D were not lysed at R.T.D. With the combined set, however, all but three of the 178 strains from all sources that were lysed only by group IV phages gave this reaction at R.T.D. With group IV/mixed strains, the situation was different. Many of the bovine-plasma coagulase-positive strains from milk were typable only at R.T.D. × 1000 with the basic-set phages, but most were typable at R.T.D. by the combined set (Table 3). Among the human strains, however, over half of all the group IV/mixed strains (44 of 71) were untypable at R.T.D. with the combined set (Table 5).

Table 6. *Number and percentage of bovine and of human strains of* Staphylococcus aureus *reacting with each of the group IV phages*

Phage no.	Bovine strains	Human strains
102	240 (78·2)	24 (3·9)
107	231 (75·2)	11 (1·8)
108	220 (71·7)	7 (1·1)
111	242 (78·8)	46 (7·5)
42F	122 (39·7)	38 (6·2)
42D	68 (22·2)	8 (1·3)
Any phage	261 (85·0)	79 (12·9)
Total number examined	307	614

Table 6 shows the number of strains of human and of bovine origin that were lysed by any of the group IV phages, and the number lysed by each of the individual phages (42D, 42F, 102, 107, 108 and 111). It appeared that phage 42D was the least productive, since it lysed about 1/4 of the bovine and only 1/10 of the human strains that were susceptible to group IV phages. It must be added that the reactions with about half of the bovine strains, and with most of the human strains, occurred only at R.T.D. × 1000. The greatest range of activity was with phage 111, and most of the reactions were at R.T.D. The range of activity of the four Davidson phages 102, 107, 108, and 111 on our bovine plasma coagulase-positive strains was approximately the same.

Most of our bovine-plasma coagulase-negative *Staph. aureus* strains from cows' milk which reacted with group IV phages belonged to group IV/mixed. They were generally lysed only by a single group IV phage, and only at R.T.D. × 1000. Considered as a group, they behaved more like strains of human origin than like the bovine-plasma coagulase-positive strains.

In addition, we examined 58 strains of *Staph. aureus* from cases of bovine mastitis received from Dr Davidson of Weybridge. For the most part they were sensitive to group IV phages, but usually only to a minority of the six phages. We are inclined to attribute this to regional differences in type-distribution rather than to differences in pathogenicity.

DISCUSSION

It appears from our findings that *Staph. aureus* strains belonging to phage-group IV are imperfectly recognized when the international basic set of phages is used. According to reports in the literature (Pöhn, 1957; Pulverer, 1964; Brandis

& Morgenroth, 1965) not more than 0·5 % of human strains are allotted to phage-group IV when phage 42D is the only member of this group used in typing. We found a considerably greater proportion of the strains lysed by phages of group IV in our human material, but this was attributable to a single epidemic of wound infection and mastitis from which a number of group IV/mixed cultures were isolated. When these are eliminated the proportion is decreased to 0·6 %. The proportion of strains from foodstuffs which react with group IV phages is rather variable; according to our experience (Meyer & Rische, 1966) it is influenced to a large extent by the percentage of milk and dairy products in the material examined.

Most authors agree that *Staph. aureus* of phage-group IV is most frequently found in cattle. Among the bovine strains we examined, about 1/4 reacted with phage 42D, but half of these were also lysed by phages of other groups. The application of the combined phage set led to an astonishing change in the grouping of the bovine organisms, and virtually all the bovine-plasma coagulase-positive strains reacted with one or more of the group IV phages. Our earlier work with bovine staphylococci, on the coagulase reaction with various kinds of plasma (Meyer, 1965, 1966*a*, *b*, *c*) and on the crystal-violet test (Meyer, 1967*a*, *b*) suggests that bovine-plasma coagulase-positive strains form a special variety of the species *Staph. aureus* which we have called var. *bovis*. Strains from milk which are bovine-plasma coagulase-negative are rather more like human staphylococci, and for this reason we consider them to be bovine only with reservation. It now appears that nearly all *Staph. aureus* var. *bovis* belong to phage-group IV or to the IV/mixed groups, and that other strains of *Staph. aureus* react much more rarely with group IV phages.

This conclusion could not have been reached had phage 42D been the only group IV phage in the typing set. With this phage we should have recognized at most 26·1 % of the bovine and only about 10 % of the human strains which react with group IV phages. It is true that by using the combined set of phages an increased proportion of human strains appears to be related to phage-group IV, but their number is not large. Most of the human strains with group IV/mixed patterns are recognized only at R.T.D. × 1000, and generally do not seem to be of great epidemiological significance.

Often, when strains reacting with group IV phages are found in human material it is supposed that they are of bovine origin and may have been transmitted from cattle to man. This must not be taken as proven. It is interesting that, according to Markuse (1960), Vogelsang, Wormnes & Östervold (1962) and Pulverer & Fritsche (1965), human group IV strains are generally egg-yolk positive. In our experience, the great majority of bovine *Staph. aureus* strains are egg-yolk negative.

The problem of establishing standardized systems for the phage-typing of *Staph. aureus* strains in a number of different animal hosts is a difficult one. A single system for all *Staph. aureus* strains is probably impracticable. It is suggested that the international basic set should be used for typing strains of human origin, and that some such set as that proposed by Davidson (1961), which includes a

number of group IV phages, should be used for bovine staphylococci. In investigations of the possible inter-specific spread of infection, it may be necessary to use both sets of phages.

SUMMARY

Because the international basic set of staphylococcus typing phages includes only one member of group IV—phage 42D—the group is insufficiently characterized. This set of phages is therefore not suitable for typing bovine strains of *Staph. aureus*. The addition of several further group IV phages would remedy this deficiency.

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Immunization of man with typhoid and cholera vaccine. Agglutinating antibodies after intracutaneous and subcutaneous injection

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INTRODUCTION

Tuft, the originator of intracutaneous typhoid immunization (Tuft, Yagle & Rogers, 1932), found that, in man, intracutaneous immunization with one-fifth of the normal dose resulted in as much antibody as subcutaneous immunization with the normal dose, and that the reaction to the vaccination was lower after intracutaneous immunization. These observations were confirmed for both re-vaccinations (Siler & Dunham, 1939; Longfellow & Luippold, 1940) and primary vaccinations (Valentine, Park, Falk & McGuire, 1935; Perry, 1937; Chiang & Ch'en, 1958). On the other hand, Morgan, Favorite & Horneff (1943) and Luippold (1944) found less antibody after intracutaneous immunization. The reduction of vaccination reactions obtained with intracutaneous immunization, even with a ten times concentrated vaccine (Shi, 1958), has been generally recognized, but Bardhan, Dutta & Krishnaswami (1963), using only twice the subcutaneous dose for intracutaneous vaccination, observed more severe reactions in the intracutaneous group. These more severe reactions were accompanied by higher antibody titres.

The intracutaneous method also proved satisfactory for vaccination with cholera vaccine (Singer, Weis & Hoa, 1948; Noble, 1964) and a combination of cholera and typhoid vaccine (Noble & Fielding, 1965). After preliminary investigations (Barr, Sayers & Stamm, 1959), the intracutaneous vaccination method was adopted by the British Army for routine immunization with a combined vaccine containing enteric and tetanus antigens (Noble, 1963).

In an attempt to improve the standard immunization schedule used for recruits of the Dutch Army, it was decided to investigate not only the possibilities for a simpler time schedule but also the question of intracutaneous vaccination.

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ห้องสมุด กรมวิทยาศาสตร์

MATERIAL AND METHODS

Subjects

About 1000 healthy 19- to 20-year-old men were randomly divided into eight groups. All the subjects were military recruits of the same draft and were stationed in the same army camp.

Vaccines

The cholera, typhoid, paratyphoid A, and paratyphoid B (CTAB) vaccine was a heat-killed, phenol-preserved suspension of bacteria, containing per ml.: 8000×10^6 *Vibrio cholerae* bacteria, 1000×10^6 *Salmonella typhi*, 750×10^6 *Salmonella paratyphi A* and 750×10^6 *Salmonella paratyphi B* bacteria. The same batch of this vaccine was used throughout the experiment.

The tetanus-diphtheria (TD) vaccine contained per ml.: 10 Lf tetanus toxoid and 30 Lf diphtheria toxoid, adsorbed with aluminium phosphate. The vaccines were standard commercial preparations obtained from the National Institute of Public Health, Utrecht, The Netherlands.

Dosage

All subjects received two 0·5 ml. doses of the tetanus-diphtheria vaccine in the deltoid muscle, with a 4- or 5-week interval (Table 1).

The dose of the CTAB vaccine differed for the eight groups according to the time schedule used, but chiefly according to the location of the injections. The subcutaneous injections were given in 0·5 or 1·0 ml. doses in the lower deltoid region. The intracutaneous injections were given in 0·1 or 0·2 ml. doses in the extensor surface of the forearm (Table 1). Opposite arms were used for the TD and CTAB vaccines.

At the time of the experiment the routine schedule of immunization of recruits was that of group 1.

Sera

Three weeks after the last injection with CTAB vaccine, blood was taken by venepuncture. Sera were stored at $-20°$ C.

Agglutination tests

Twofold serum dilution series beginning with 1/10 were made in volumes of 0·5 ml. in 0·9 % NaCl solution, in round-bottomed tubes of 50×7 mm. To these serum dilutions, equal volumes of bacterial suspensions were added forcibly to achieve good mixing. The first serum dilution thus became 1/20. The salmonella antigens were the standard commercial preparations for the determination of O- and H-agglutinins of the National Institute of Public Health, Utrecht, The Netherlands. Since a suitable cholera antigen for the agglutination test was not commercially available, the cholera antigen was freshly prepared each day by suspending in 0·9 % NaCl solution the 18 hr. growth on nutrient agar slopes of *Vibrio cholerae* of the Ogawa and Inaba strains (see Vella & Fielding, 1963). The

Table 1. *Time schedules, type of injection (subcutaneous = s.c., or intracutaneous = i.c.), and doses of the CTAB vaccine*

Week	1	2	3	4	5	6	7	8
Date	6. iv. 60	13. iv. 60	21. iv. 60		3. v. 60	10. v. 60	17. v. 60	27. v. 60
Group								
1	TD	—	—	—	TD	— CTAB s.c. 0·5 ml.	— CTAB s.c. 1·0 ml.	— CTAB s.c. 1·0 ml.
2	TD	—	—	—	TD	— CTAB i.c. 0·1 ml.	— CTAB i.c. 0·2 ml.	— CTAB i.c. 0·2 ml.
3	TD	— CTAB s.c. 0·5 ml.	— CTAB s.c. 1·0 ml.	—	— CTAB s.c. 1·0 ml.	TD	—	—
4	TD	— CTAB i.c. 0·1 ml.	— CTAB i.c. 0·2 ml.	—	— CTAB i.c. 0·2 ml.	TD	—	—
5	TD CTAB s.c. 0·5 ml.	—	— CTAB s.c. 1·0 ml.	—	TD CTAB s.c. 1·0 ml.	—	—	—
6	TD CTAB i.c. 0·1 ml.	—	— CTAB i.c. 0·2 ml.	—	TD CTAB i.c. 0·2 ml.	—	—	—
7	TD CTAB s.c. 1·0 ml.	—	—	—	TD CTAB s.c. 1·0 ml.	—	—	—
8	TD CTAB i.c. 0·2 ml.	—	—	—	TD CTAB i.c. 0·2 ml.	—	—	—

Table 2. *Means (m) and standard deviations (sd) of the transformed titres (log_2 of 1/10 of the reciprocal of the serum dilution)*

Group	No. of persons	antigen: TO		TH		AO		AH		BO		BH		V. CHOL.	
		m	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*
1	50	4·1	1·5	5·3	1·6	3·2	1·9	7·0	1·2	4·0	1·3	6·6	1·5	5·0	1·3
2	50	3·8	1·5	5·8	1·9	2·8	2·0	7·2	1·7	3·9	1·4	7·5	1·8	4·7	1·4
3	50	3·7	1·2	5·2	1·2	2·6	2·0	6·7	1·4	3·8	1·3	6·8	1·4	4·8	1·5
4	50	3·7	1·1	5·6	1·2	2·9	2·0	6·9	1·3	3·9	1·4	6·8	1·7	4·6	1·0
5	50	4·1	1·4	4·9	1·4	3·0	2·0	6·4	1·3	4·3	1·3	6·2	1·8	4·7	1·3
6	50	3·8	1·6	5·3	1·4	3·1	2·0	6·7	1·3	3·9	1·4	7·0	1·5	4·6	1·1
7	50	3·9	1·3	4·6	1·4	2·7	1·8	6·4	1·4	4·2	1·1	6·3	1·4	4·8	1·1
8	50	4·0	1·4	5·3	1·3	2·9	2·0	6·9	1·3	4·1	1·4	6·9	1·5	4·7	1·0

Addition to Table 2 from the literature: agglutination titres from field trials, expressed in the same transformations as our data

	No. of persons	TO *m*	TO *sd*	TH *m*	TH *sd*	
Acetone	217	0·7	0·9	6·7	1·4	Data from Ashcroft *et al.* (1964)
Heat-phenol	207	0·9	0·9	6·1	1·9	
Alcohol	200	2·3		5·6		Data from Yugoslav typhoid commission (1962)
Heat-phenol	200	2·6		5·8		

resulting suspension contained about 5×10^8 bacteria per ml. Strains were selected for smoothness by their resistance to complement (Singh & Ahuja, 1951).

After 18 hr. incubation at 37° C. in a water-bath, the agglutinations were read with the naked eye by the pattern on the bottom of the tube, without shaking the tubes. The serum dilution in the last tube showing agglutination as compared to the control tube, was taken as the titre of the serum. This corresponds to the 'last trace' reading of Gardner (1937), Felix (1938), and Vella (1963).

To equalize possible daily variation, care was taken to investigate equal numbers of sera from each of the eight groups each day. To avoid bias in reading the agglutination tests, sera were coded so that the investigator would be unaware of the group number of the sera when reading. All agglutination tests were read by the same investigator. The investigations were terminated when, for each group, the sera of 50 persons who had completed the immunization schedule had been examined.

RESULTS

For convenience of calculation and presentation, the reciprocals of the titres were divided by 10 and the logarithms to the base 2 of these numbers were taken. The values of the sera thus became the same as the numbers of the last tubes in which agglutination was observed. These values will be called transformed titres. For each antigen and each group, the cumulative frequency distribution of the transformed titres, plotted on normal probability paper, approached a straight line sufficiently closely for the frequency distribution of the transformed titres to be considered normal. Therefore, the results could be evaluated by comparing the means and standard deviations of the transformed titres (see Table 2). Differences in the means of groups 1 and 8, evaluated with the *t*-test, did not reach the 1 % level of significance.

An attempt to compare the incidence of reactions after intracutaneous and subcutaneous injection failed, because the number of reactions recorded by the doctors (nine out of 550 subcutaneous injections and five out of 550 intracutaneous injections) was too low to show significant differences. Calculations according to Lidwell (1963) and Kheifetz & Khazanov (1959) showed that at least 150 persons per group (i.e. 1650 injections of each kind) would have been necessary to obtain a significant 50 % reduction.

No local reactions were reported.

DISCUSSION

As shown by field trials, the mouse is not a good experimental model for the selection of typhoid vaccines for use in man (Standfast, 1964). Probably no better guide for the selection of an effective typhoid vaccine is provided by the titres of agglutinating antibodies in human sera after immunization (see addition from the literature in Table 2), although differences between agglutination titres produced by more effective and less effective vaccines are statistically significant (Benenson, 1964). Nevertheless, once a vaccine is chosen, we think that the agglutination test has some place in solving minor problems such as we were dealing with in this study.

The special role of the skin in immunologic processes was stressed long ago (Zinn & Katz, 1927; Tuft, 1931). For inducing allergic processes, the effect of injecting intracutaneously has even been called equal to that of using adjuvants (Waksman, 1956; see also Leskowitz & Waksman, 1960). The particular effectiveness of the cutis as an immunization route is possibly connected with the abundance of the lymphatic network of the corium as compared to that of the subcutis (Rusznyak, Foldi & Szabo, 1960). Intracutaneous injection has been described as an intralymphatic injection (Hudack & McMaster 1933), but the fate of the injected fluid under the influence of such variables as tissue pressure and inflammation is not clear (McMaster & Parsons 1950; Rusznyak, Foldi & Szabo 1960). In any case, the absorption of an antigen differs according to whether it concerns a primary or a secondary intracutaneous injection (Birkhaug & Boe, 1946; Korngold *et al.* 1953).

When no definite information on the essential difference between the subcutis and the cutis as a route of immunization could be obtained from the literature, another aspect of this problem suggested itself, namely that of the antigen-dose/antibody-response relationship. The literature survey done by Stevens (1956) (see also Stille, 1960) gives a general impression of this relationship, but we still did not feel certain that with one-fifth of the normal dose of CTAB vaccine injected subcutaneously we would not have obtained the same titres as with the full dose. Tuft (1931) injected the same dose intracutaneously, subcutaneously and intramuscularly, but the results led him to remark that 'it is rather difficult to give subcutaneous and intramuscular injections without involvement of the skin'. This same difficulty was mentioned by Waksman & Morrison (1951). The failure in our experiments to try the effect of the smaller intracutaneous doses given subcutaneously made it necessary to undertake further investigation of the antigen-dose/antibody-response relationship with typhoid vaccine in man (Clasener, 1967).

CONCLUSION

No reason was found, with respect to agglutinating antibody titres or vaccination reactions, for not simplifying the rather laborious immunization schedule of group 1 into the much simpler schedule of group 8 for the routine immunization of recruits.

SUMMARY

Young men were immunized against cholera, typhoid, paratyphoid A, and paratyphoid B. Agglutinating antibodies were measured 3 weeks after completion of the immunization schedule. Two injections separated by a 4-week interval were found to be as efficient as three injections with 1-week intervals.

Intracutaneous immunization with one-fifth of the standard subcutaneous dose was just as efficient as the standard subcutaneous immunization and did not cause greater general or local reaction.

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Immunization of man with salmonella vaccine and tetanus–diphtheria vaccine. Dose-response relationship, secondary response, and competition of antigens

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INTRODUCTION

During an investigation into intracutaneous typhoid immunization (Clasener & Beunders, 1967) we encountered the problem of the dose-response relationship in the immunization of man with typhoid vaccines. Since the literature (see Stevens, 1956) contained no relevant data on the combination of antigen, antigen dose, and species we were interested in, an experiment seemed necessary. In this work, the standard immunization schedule for military recruits was changed only in that some groups were given less than the normal amount of salmonella antigen. The diphtheria and tetanus immunization, given simultaneously in this schedule, was the same for all test groups. This also offered an opportunity to determine the influence of the different doses of salmonella vaccine on the antibody response to tetanus–diphtheria immunization.

If an organism is stimulated by several antigens simultaneously, the production of antibody for one antigen may be influenced by the stimulation with the other antigens, this influence being called synergy if positive and competition if negative. Both kinds of influence undoubtedly exist (Adler, 1959; Johnson, 1964), but are dependent on many variables such as the kind of antigen, the absolute and relative amounts of antigen, previous contact with antigens, species, etc. (Adler, 1964). Immunization procedures in man are not always well founded in this respect (see Barr & Llewellyn-Jones, 1953).

MATERIALS AND METHODS

Subjects

About 200 healthy 20-year-old males were randomly divided into four groups.

Antigens and dosage

Tetanus–diphtheria (TD) vaccine contained tetanus toxoid 10 Lf/ml. and diphtheria toxoid 5 Lf/ml., adsorbed with aluminium phosphate. Typhoid-paratyphoid (TAB) vaccine contained *Salmonella typhi* 1000×10^6/ml., *S. paratyphi A* 750×10^6/ml. and *S. paratyphi B* 750×10^6/ml. The vaccine was killed and preserved with acetone.

The same batch of each vaccine was used throughout the experiment. The vac-

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cines were standard commercial preparations of the National Institute of Public Health, Utrecht.

Immunization schedule

Table 1 shows the detailed schedule. It is seen that the doses of TAB differed for the four groups, but all groups received the same dose of TD. The two vaccines were given in opposite arms.

Military recruitment in The Netherlands is normally the first occasion for vaccination against typhoid, so the TAB vaccination was certainly a primary one for all subjects; the TD vaccination might have been secondary for some.

Table 1. *Immunization schedule in ml. for the two vaccines*

	5. ii. 65		4. iii. 65	
Group	TD*	TAB†	TD*	TAB†
1	0·5	—	0·5	—
2	0·5	0·1	0·5	0·1
3	0·5	0·2	0·5	0·2
4	0·5	1·0	0·5	1·0

* Given intramuscularly. † Given subcutaneously.

Sera

Blood was taken by venepuncture at the time of the first and second injections, and 3 weeks after the second injection. Sera were stored at $-20°$ C.

Antibody titrations

Antibodies for the salmonella antigens were measured by the bacterial agglutination method. Antibodies for tetanus and diphtheria antigens were measured by the passive haemagglutination technique (Stavitsky, 1954; Tasman, van Ramshorst & Smith, 1960).

Bacterial agglutinations

Twofold serum dilution series beginning with 1/10 were made in volumes of 0·5 ml. in 0·9 % NaCl solution. Round-bottomed test tubes (50 × 7 mm.) were used. To these serum dilutions, equal volumes (0·5 ml.) of killed bacterial suspensions were added, resulting in serial twofold serum dilutions beginning with 1/20.

The bacterial suspensions were standard commercial preparations for the determination of O- and H-agglutinins, obtained from the National Institute of Public Health, Utrecht, The Netherlands. After incubation overnight at room temperature, covered with a plastic sheet to prevent evaporation, readings were made under a strong light from above against a black background with a binocular headloupe giving a 2·25 magnification.

All sera taken on the three dates from all subjects who had received TAB vaccine were titrated on the same day against the same batch of antigen for each of the antigens used. Sera of group 1 were not titrated for salmonella agglutinins.

The batch of *Salmonella paratyphi A* O antigen was not suitable for use.

Scoring

The number of the last tube showing agglutination, as compared to the control tube, was taken as the value of the serum. This is the same as taking the $\log_2$ of 1/10 of the reciprocal of the serum dilution. The sera showing no agglutination in the first dilution were assigned a value of 0.

Haemagglutination

Erythrocytes

Sheep blood was collected in equal volumes of Alsever solution. After centrifugation, the red cells were washed three times in saline buffered at pH 7·4 by mixing equal volumes of 0·15 M-NaCl and 0·15 M phosphate buffer (pH 7·4). The packed cells were stored at 4° C. for 3 days with 1/10,000 merthiolate, washed once to be sure there was no lysis, and then used for adsorption or sensitization.

Adsorption

To remove any sheep red-cell antibodies, all sera were adsorbed with sheep red cells. Four volumes of a 25 % suspension of sheep red cells were incubated overnight at 4° C. with 1 vol. of heat-inactivated serum. After centrifugation, the resulting 1/4 serum dilution was stored at −20° C.

Sensitization

Equal volumes of a 2 % red-cell suspension and a 1/40,000 solution of tannin (BDH) in saline were mixed at room temperature for 10 min. After centrifugation and one washing with 0·15 M-NaCl, the cells were resuspended in 0·15 M-NaCl to the original volume. This tanned 2 % red-cell suspension was then mixed with an equal volume of toxoid solution containing 30 Lf diphtheria or tetanus toxoid per ml. of saline buffered at pH 6·4 by mixing equal volumes of 0·15 M-NaCl and 0·15 M phosphate buffer (pH 6·4). After centrifugation, the sensitized cells were washed three times and resuspended in 1 % rabbit serum in saline. Rabbit blood was obtained by cardiac puncture. After being held for one hour at 37° C. and overnight at 4° C., serum was pipetted off and stored at −20° C. On the day of use, the rabbit serum was heat-inactivated and diluted 1/100. The 2 % suspension of sensitized cells in 1 % rabbit serum was kept at 4° C. for 3 days, within which period all sera were titrated. All sera taken on the three dates were titrated with the same batch of sensitized cells. All sera taken on the same date were titrated on one day. This was done to exclude, as far as possible, variations in the properties of the antigen and daily variations in the titration of the sera to be compared.

Dilutions

Twofold serum dilution series beginning with 1/10 were made in volumes of 0·5 ml. in 1 % rabbit serum. Plastic plates with round-bottomed cups 15 mm. in diameter were used. To these serum dilutions the sensitized red cells were added as equal volumes (0·5 ml.) of a 1/10 dilution, in 1 % rabbit-serum, of the 2 % suspension. Readings were made after overnight incubation at room temperature.

Scoring

The number of the last cup in which the maximal reaction was seen, was taken as the value of the serum. With a serum dilution of 1/20 in the first cup, this way of scoring amounts to taking $\log_2$ of 1/10 of the reciprocal of the serum dilution. Sera negative in the first dilution were assigned a value of 0. The logarithmic transformation makes the resulting frequency distributions symmetrical instead of skewed; the other transformations simplify the calculations.

Control sera containing 10 AU diphtheria or tetanus antitoxin per ml. scored 9. So with this system we could measure a minimum antibody content of 0·04 AU per ml.

RESULTS

Results of the bacterial agglutinations

These are shown in Table 2. As can be seen in the graph (Fig. 1) constructed from the data in this table, the dose-response correlation can be roughly described with the formula of Smith & St John-Brooks (1912): log dose = $k + 1/n$ log titre.

Table 2. *Means and standard deviations (sd) of the 'titres' (transformation see text) of the agglutinating bacterial antibodies before and after vaccination with various doses of TAB vaccine*

		Group 2 (0·1 ml.) 44 subjects		Group 3 (0·2 ml.) 36 subjects		Group 4 (1·0 ml.) 43 subjects	
Antigen	Date	Mean	*sd*	Mean	*sd*	Mean	*sd*
TO	5. ii. 65	1·6	1·4	1·5	1·3	1·0	1·4
	4. iii. 65	2·8	1·2	3·5	1·1	4·9	1·0
	26. iii. 65	2·8	1·2	3·1	1·1	4·3	1·0
BO	5. ii. 65	1·8	1·2	1·6	1·0	1·0	1·2
	4. iii. 65	2·4	1·1	2·5	1·0	3·5	1·1
	26. iii. 65	2·1	1·3	2·5	1·0	3·0	1·0
TH	5. ii. 65	0·0	—	0·0	—	0·0	—
	4. iii. 65	4·4	2·0	4·9	1·6	5·8	1·3
	26. iii. 65	3·8	1·9	4·7	2·1	5·3	1·2
AH	5. ii. 65	0·0	—	0·0	—	0·0	—
	4. iii. 65	5·3	1·4	5·8	1·2	6·3	1·0
	26. iii. 65	4·7	1·4	5·1	1·1	6·3	1·1
BH	5. ii. 65	0·0	—	0·0	—	0·0	—
	4. iii. 65	3·9	1·3	4·9	1·4	5·7	1·1
	26. iii. 65	3·7	1·0	4·5	1·5	6·0	1·0

Some antibody against TO- and BO-salmonella antigens was found in the pre-immunization sera taken on 5 Feb. 1965.

No enhanced secondary response was seen after the second TAB injections as judged from a comparison of the values reached on 4 Mar. 1965 (4 weeks after the first TAB injection) and 26 Mar. 1965 (3 weeks after the second TAB injection).

Results of the toxoid haemagglutination tests

The influence of the primary TAB immunization on the tetanus-diphtheria immunization may depend on whether the latter was primary or secondary. Since the system used was not very sensitive, absence of antibodies in the sera taken before

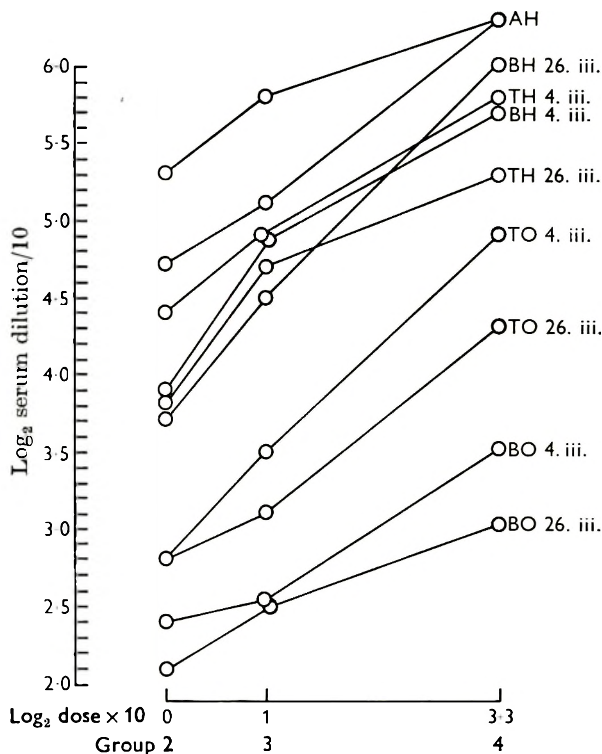

Fig. 1. Antigen-dose/antibody-response relationship for the three groups shown in Table 2.

immunization would not exclude previous contact with the antigen. From the bimodality of the frequency distribution of the titres of the four groups together (left part of Tables 3 and 4) it seemed justifiable to assume that absence of antibodies in the sera taken 4 weeks after the first injection indicated a primary response. On the basis of this definition of primary response, the bimodal frequency distribution of tetanus antibody titres of the sera taken 3 weeks after the second injections (26 Mar. 1965), could be nicely split, resulting in four classes. The class of persons who reacted with a secondary response to both diphtheria and tetanus antigens had probably been immunized previously (Table 5). Persons who had a secondary response to the diphtheria antigen and a primary response to the tetanus antigen had probably had their contact with the diphtheria antigen by infection, because in childhood tetanus and diphtheria vaccine are usually given together (Table 6). Only a few subjects showed a primary response to both tetanus and diphtheria antigens (Table 7). None showed a primary response to diphtheria antigen

Table 3. *Classification of the total population according to the tetanus antitoxin level on* 4 *March* 1965

Values of the sera expressed as the number of the last positive cup	Frequency distribution of all sera of the 4 groups			Frequency distribution of the sera of all subjects of the 4 groups whose tetanus vaccination was probably: Secondary			Primary		
	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65
0	119	84	5	35	—	—	84	84	5
1	13	—	—	13	—	—	—	—	—
2	12	2	1	12	2	—	—	—	1
3	9	3	6	9	3	1	—	—	5
4	4	4	27	4	4	—	—	—	27
5	5	1	28	5	1	2	—	—	26
6	7	14	16	7	14	2	—	—	14
7	1	18	7	1	18	4	—	—	3
8	—	23	9	—	23	7	—	—	2
9	—	12	14	—	12	13	—	—	1
10	—	6	15	—	6	15	—	—	—
11	—	2	10	—	2	10	—	—	—
12	—	1	19	—	1	19	—	—	—
13	—	—	7	—	—	7	—	—	—
14	—	—	1	—	—	1	—	—	—
15	—	—	—	—	—	—	—	—	—
16	—	—	5	—	—	5	—	—	—
Total	170	170	170	86	86	86	84	84	84

Table 4. *Classification of the total population according to the diphtheria antitoxin level on* 4 *March* 1962

Values of the sera expressed as the number of the last positive cup	Frequency distribution of all sera of the 4 groups			Frequency distribution of the sera of all subjects of the 4 groups whose diphtheria vaccination was probably: Secondary			Primary		
	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65	5. ii. 65	4. iii. 65	26. iii. 65
0	88	16	13	72	—	—	16	16	13
1	3	—	—	3	—	—	—	—	—
2	10	3	1	10	3	—	—	—	1
3	16	1	1	16	1	—	—	—	1
4	23	8	3	23	8	3	—	—	—
5	15	14	9	15	14	9	—	—	—
6	10	31	8	10	31	8	—	—	—
7	1	36	11	1	36	10	—	—	1
8	3	48	17	3	48	17	—	—	—
9	1	7	24	1	7	24	—	—	—
10	—	4	23	—	4	23	—	—	—
11	—	1	11	—	1	11	—	—	—
12	—	1	20	—	1	20	—	—	—
13	—	—	1	—	—	1	—	—	—
14	—	—	4	—	—	4	—	—	—
15	—	—	1	—	—	1	—	—	—
16	—	—	23	—	—	23	—	—	—
Total	170	170	170	154	154	154	16	16	16

together with a secondary response to the tetanus antigen. Some values of groups 1 and 4 were compared by means of Student's test. Values of t are given in Tables 5 and 6. These differences are not significant at the 1 % level. Furthermore, no significant differences were found for the total values (not given) of each group or

Table 5. *Number (n), mean (m) of the antitoxin value and standard deviation (sd) of the class of subjects of each group for whom the tetanus and diphtheria immunization were probably secondary*

	5. ii. 65			4. iii. 65			26. iii. 65		
Group	*n*	*m*	*sd*	*n*	*m*	*sd*	*n*	*m*	*sd*
					Diphtheria				
1	28	2·7	2·2	*28	7·4	1·4	28	10·4	3·8
2	21	2·7	2·8	21	6·7	1·7	21	9·9	3·2
3	17	3·0	2·3	17	6·8	1·5	17	10·2	2·7
4	20	2·6	2·9	*20	6·6	1·1	20	10·6	3·3
					Tetanus				
1	28	1·5	2·1	28	7·3	2·2	†28	10·5	2·8
2	21	2·0	2·0	21	7·3	1·8	21	10·3	2·1
3	17	1·7	2·3	17	6·8	1·9	17	9·8	3·2
4	20	2·1	1·8	20	7·8	1·7	†20	11·1	1·7

* $t = 2{\cdot}24$. † $t = 0{\cdot}87$.

Table 6. *Number (n), mean (m) of the antitoxin value and standard deviation (sd) of the class of subjects of each group for whom the diphtheria immunization was probably a secondary one and the tetanus immunization probably a primary one*

	5. ii. 65			4. iii. 65			26. iii. 65		
Group	*n*	*m*	*sd*	*n*	*m*	*sd*	*n*	*m*	*sd*
					Diphtheria				
1	16	1·2	1·7	*16	7·6	1·5	†16	11·5	3·7
2	19	1·3	2·1	19	7·1	2·0	19	10·4	3·0
3	15	1·1	1·7	15	5·7	2·2	15	8·5	3·4
4	18	2·3	2·4	*18	6·8	1·3	†18	9·9	2·7
					Tetanus				
1	16	0·0	—	16	0·0	—	‡16	5·4	1·3
2	19	0·0	—	19	0·0	—	19	4·9	1·7
3	15	0·0	—	15	0·0	—	15	3·8	1·6
4	18	0·0	—	18	0·0	—	‡18	4·2	1·9

* $t = 1{\cdot}67$. † $t = 1{\cdot}38$. ‡ $t = 2{\cdot}10$.

for the number of subjects in each group located in the same class of response type. Comparison of the diphtheria antitoxin values in Tables 5 and 6 shows clearly that the secondary response to diphtheria vaccination is not influenced by the factor of whether the tetanus immunization is a primary or a secondary one.

Table 7. *Number (n), mean (m) of the antitoxin value of the class of subjects of each group for whom the tetanus and diphtheria immunization were probably primary*

	5. ii. 65		4. iii. 65		26. iii. 65	
Group	*n*	*m*	*n*	*m*	*n*	*m*
			Diphtheria			
1	3	0·0	3	0·0	3	0·0
2	4	0·0	4	0·0	4	0·0
3	4	0·0	4	0·0	4	2·5
4	5	0·0	5	0·0	5	0·4
			Tetanus			
1	3	0·0	3	0·0	3	4·7
2	4	0·0	4	0·0	4	5·3
3	4	0·0	4	0·0	4	4·8
4	5	0·0	5	0·0	5	3·8

DISCUSSION

Salmonella antibodies

Intracutaneous TAB immunization of man with 0·2 ml. doses results in agglutinating antibody titres as high as those obtained with 1·0 ml. doses given subcutaneously (Clasener & Beunders, 1967). The dose-response correlation found in this study demonstrates that this result is dependent on the intracutaneous route: when 0·2 ml. doses are given subcutaneously, the titres are considerably lower than when 1·0 ml. doses are given subcutaneously.

Since TAB immunization in The Netherlands is not normally given before military service, the antibodies in the prevaccination sera against the TO- and BO-antigens probably resulted from natural contact.

Whether the response after a second antigenic stimulation will be much higher than after the primary stimulation depends on several factors. Besides the dose of primary stimulation (Uhr & Finkelstein, 1963) and time interval (Fecsik, Butler & Coons, 1964), the most important factor is probably the kind of antigen. With particulate microbial antigens there is no difference between primary and secondary response such as is found with toxoids (Burnet & Fenner, 1949; Bauer, Mathies & Stavitsky, 1963). The absence of a secondary response to salmonella vaccine probably also explains the fact that in our first experiment (Clasener & Beunders, 1967) the group that received three injections did not produce more agglutinating antibodies than the group that received two injections. It seems very important in this connexion that in field trials the protection after one injection of typhoid vaccine was found to be as high as that after two injections (Yugoslav Typhoid Commission, 1964; Ashcroft, Ritchie & Nicolson, 1964).

Tetanus and diphtheria antibodies

Although the haemagglutination technique does not recognize one possibly important property of antitoxins, namely avidity, the correlation between haemagglutination titres and toxin-neutralizing antibodies seems sufficiently well estab-

lished to give biological significance to the results (Scheibel, 1956; Tasman *et al.* 1960; Surján & Nyerges, 1962*a*, *b*).

Whereas the titres of salmonella agglutinating antibodies were divergent for the four groups, correlating with the different doses, haemagglutinating antibody titres against diphtheria and tetanus were essentially the same for the four groups. It appears therefore that, in men of this age, immunization with salmonella antigens has no influence on a simultaneous primary or secondary immunization against diphtheria or tetanus.

SUMMARY

Immunization of men with various amounts of salmonella antigens gave a linear relationship between the logarithm of the dose and the logarithm of the titres of the antibodies produced. No secondary response was observed for any of the doses used. These doses of salmonella vaccine did not interfere with or stimulate simultaneous primary or secondary vaccination with tetanus and diphtheria vaccine.

This experiment would not have been possible without the generous cooperation of Colonel B. J. W. Beunders, M.D., Head of the Health Department of the Dutch Army and Airforce, and his collaborators. I am also greatly indebted to Prof. Dr J. E. Dinger for his stimulation and help. Discussion with Dr A. Tasman on the design of the experiment, with Dr G. L. Smit on the haemagglutination method and Dr H. A. Valkenburg on the statistical aspects of the study are gratefully acknowledged. Dr J. D. van Ramshorst kindly provided the diphtheria and tetanus toxoids and the control sera for the haemagglutination reaction.

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A morphological comparison of Bittner and influenza viruses*

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It has been known for some time that negatively stained influenza virus and Bittner mouse mammary tumour virus resemble each other (Almeida & Ham, 1965). However, negative staining of intact virus delineates only the surface features of the particle and it seems possible that this external similarity does not extend to the inner components of these viruses, as they differ considerably both in physical properties and in their thin sectioned appearance (Moore, 1962; Morgan, Rose & Moore, 1956). Thin sections of mouse mammary tumours reveal two distinct forms which have been designated as virus (the types A and B of Bernhard) (Bernhard, 1958), while cells infected with influenza virus show only one form recognizable as virus, seen always at the surface of the cell and quite distinct from the type B Bittner particles. The present paper describes the results obtained when negative staining was used to study the internal component of both these viruses in particles that could be penetrated by the phosphotungstic acid (PTA). In addition, the negatively stained appearance of the mouse mammary tumour virus was compared with the picture that is obtained when the virus is studied by the thin sectioning technique.

MATERIALS AND METHODS

Bittner virus

Female C3H mice with spontaneously occurring mammary tumours were used as a source of Bittner virus. The animals were killed at ages varying from 9 to 18 months. The tumours were removed, and portions fixed in 10% formol saline for sectioning for light microscopy and in buffered osmium tetroxide for electron microscopy. Blocks for thin sectioning were embedded in Araldite according to conventional techniques (Luft, 1961). The remainder of the tumour was either used immediately for making extracts or frozen at $-20°$ C. for periods of up to 6 months. Extracts were made by homogenizing the tumours in a glass homogenizer of the tenBroek type using distilled water as a suspending medium. The suspension was spun at 10,000 rev./min. for 10 min. in the SS34 rotor of the Sorvall RC-2 centrifuge, and the supernatant was then spun for 30 min. at

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15,000 rev./min. in the same rotor. This pellet was suspended in a small amount of distilled water (approx. 0·2 ml.) and negatively stained by mixing a small amount of this suspension with an equal quantity of 3 % phosphotungstic acid adjusted to pH 6. Carbon–formvar coated grids of 400-mesh were used and the specimens examined in a Philips EM200 electron microscope.

Influenza virus

The virus was grown by inoculating the PR 8 strain of influenza A virus into the allantoic cavity of 11-day embryonated eggs. After 3 days incubation the eggs were chilled and the allantoic fluid harvested. Some of this was used immediately to obtain a virus pellet while other portions were placed in vials and frozen at −20° C. for periods of up to 6 months. After a clarifying spin of 3000 rev./min. for 5 min., the supernatant was centrifuged for 30 min. at 15,000 rev./min. in the SS34 rotor of the Sorvall RC-2 centrifuge. The pellet was resuspended in distilled water and negatively stained as described for Bittner virus.

RESULTS

Light microscopy

Examination of sections stained with haematoxylin and eosin in the light microscope confirmed that the tumours displayed the histopathology associated with them (Moore, 1962). Duct formation was frequently seen, and the cells lining these ducts often displayed the eosinophilic juxtanuclear inclusions characteristic of the Bittner tumour.

Thin sectioning

Examination of thin sections of the mouse mammary tumours in the electron microscope again confirmed the features associated with such tumours (Moore, 1962). Type A particles, approximately 700 Å. in diameter, were frequently found in areas near membranes where type B particles, approximately 1000 Å. in diameter, were budding (Pl. 1, fig. 1). In several instances the morphology of the free type A particles could be seen within budding particles (Pl. 1, figs. 1, 2). Both immature (Pl. 1, fig. 2) and mature (Pl. 1, fig. 3) type B particles were present.

Negative staining

Bittner virus

Although the age of the mice at the time the tumour was taken differed by as much as 9 months and the state of the tumours varied from well differentiated to anaplastic, negative staining revealed virus particles in all the tumour extracts in a series of 25 (Pl. 2, fig. 4). The particles seen were identical with those described previously by others (Lyons & Moore, 1962; Parsons, 1963*a*). They were pleomorphic in form, generally in the size range 800–1000 Å., were bordered by a fringe 70 Å. long, and, although essentially pleomorphic, one particular form was frequently observed. This was a tailed particle with a head of approximately 1000 Å. and a tail of up to 3000 Å. in length (Pl. 3, fig. 6). Initially, no internal component was observed in the extracts of freshly excised tumours. However, tumours stored for periods of up to 6 months at −20° C., when examined by negative

staining, were found to contain degraded particles whose number increased with time of storage. The envelope of such particles was penetrated by the phosphotungstic acid and this revealed the presence of an internal circular component (Pl. 3, fig. 8; Pl. 4, figs. 10, 11). Of the many degraded particles seen the outline of the internal body was always circular, and since the centre usually appeared darker than the rim it might be presumed to be a collapsed sphere. This central spherical body measured between 750 and 800 Å. in diameter and could be identified with the type A particles seen previously in negatively stained cell-spread preparations (Parsons, 1963*b*). This internal component revealed some surface structure in the form of poorly defined subunits, although these did not appear to be regularly arranged (Pl. 3, fig. 9). In places at the edge of the central body an appearance compatible with a two-layered structure could be seen (Pl. 3, fig. 9). Neither in general shape nor in subunit arrangement did the Bittner internal component resemble the capsid of any virus known to have cubic symmetry. The internal body was frequently seen in a state of disintegration (Pl. 4, fig. 10), but when this occurred no further structure was revealed. Disintegrating type A particles had a greater diameter than the intact forms (Pl. 4, fig. 10). Occasionally free-lying type A particles were seen in these preparations (Pl. 3, fig. 9).

Influenza virus

Pellets of the PR 8 strain of influenza when negatively stained revealed typical virus particles which, like Bittner virus, would be described as pleomorphic and having a distinctive fringe (Horne *et al.* 1960). The fringe on influenza virus was longer than that of the Bittner particles, measuring 100 Å. (Pl. 2, fig. 5). Filamentous forms were frequently observed but these could not be confused with the tailed particles described for the mouse mammary tumour virus, for although there is frequently a swelling at the ends of the influenza filaments they are not of the same dimension as the heads of the tailed Bittner virus particles (Pl. 3, fig. 7). Influenza particles in which the internal component was visible were not immediately identified on viewing these preparations.

Specimens of influenza virus that had been stored for varying times at $-20°$ C. were examined in the microscope, but unlike Bittner did not seem to lead to any increase in the number of penetrated particles. During this time it was discovered, almost accidentally, that if any of these influenza specimens, either fresh or frozen, were scanned with diligence a very small number of particles revealing internal structure could be found (Pl. 3, fig. 7). It was found that if a preparation of approximately 5×10^4 haemagglutinating units/ml. was used to prepare the grid then half an hour's scanning time at the electron microscope would reveal an average of two particles in which the internal component could be seen. This meant that we could establish a roughly quantitative basis for the assessment of breakdown in influenza virus preparations, and allowed us to confirm by a more objective means that storage at $-20°$ C. had not altered the influenza virus population.

It might be remarked that after establishing this finding a re-examination of freshly extracted Bittner virus particles showed that there too a small number of spontaneously degraded particles were present.

As has been previously described (Waterson, Hurrell & Jensen, 1962; Lovas & Takatsy, 1965; Apostolov & Flewett, 1965; Klimenko *et al.* 1966), the internal component of influenza virus is a coil formed from a strand approximately 60 Å. in diameter. According to our measurements the diameter of the whole coil varied between 400 and 600 Å. with the majority being close to 500 Å. It was only infrequently that a coil was sufficiently resolved for the number of turns in it to be counted, but in the most favourable instances the number of turns was found to be close to 10. In both influenza and Bittner virus preparations particles were observed that contained more than one internal component (Pl. 4, figs. 11, 12).

DISCUSSION

Until now it has been presumed that virus particles having similar external appearances might be expected to contain internal components belonging to the same symmetry group. For example, the many different types of para-influenza viruses, Newcastle disease virus, mumps, and the measles–rinderpest–distemper group, although differing in many of their properties, have within their similar envelopes indistinguishable helical structures. This has been established readily, as all of these viruses disrupt spontaneously and allow examination of the internal component in any negatively stained electron microscope specimen. However, Bittner and influenza viruses have not yielded so easily to study, because they are not spontaneously disrupted, and treatment with organic solvents, although disintegrating the virus, does not allow the internal component to be readily seen (Hoyle, Horne & Waterson, 1961; Lyons & Moore, 1965). The technique used in this study (storage at $-20°$ C.) was most successful in revealing the internal component of Bittner virus, but unsuccessful with influenza. However, a small number of spontaneously disrupted particles of influenza virus were eventually found, making it possible to carry out a comparative study of the two internal components.

It was rather surprising to find that influenza and Bittner virus, although externally similar, contained totally different internal components. It is taken for granted that as the influenza internal component is in the form of a strand of ribonucleoprotein (RNP) it will have underlying helical symmetry, although this has not as yet been satisfactorily resolved in the electron microscope. For this reason influenza virus is generally grouped with those viruses having compound helical structure (Waterson & Almeida, 1966). On the other hand, Bittner virus, also an RNA virus (Lyons & Moore, 1965), would now appear to have a spherical internal component. Until now a virus having an apparently spherical nucleocapsid has almost always been shown in the electron microscope to be built of regular, repeating sub-units arranged to give cubic symmetry (Almeida, 1963). We have not been able to show any regularity in the arrangement of the rather poorly defined subunits forming the internal component of Bittner virus, neither is there any suggestion of the hexagonal outline associated with cubic viruses. This may be because Bittner virus has a type of morphology not previously encountered in an animal virus; on the other hand, it may be that the Bittner particle does belong to

one of the two main virus symmetry groups, cubic or helical (Caspar & Klug, 1962), but we have not been able to see the symmetry-bearing component. For example there may be a ribonucleoprotein helix within this spherical component or there could be cubic symmetry basic to the surface structure that we have seen on the spherical component. It is interesting that when the superficially similar Rauscher mouse leukaemia virus was examined by negative staining, one group of workers (de-Thé & O'Connor, 1966) interpreted their micrographs as revealing helical structures while another group using snake venom to degrade the particles demonstrated a cubic component (Padgett & Levine, 1966).

Although the Rauscher virus does not have the distinctively fringed outer envelope exhibited by Bittner virus the overall morphology of the two particles is similar (Padgett & Levine, 1966). This gives rise to the rather interesting speculation that although the events of recent years, and most particularly those concerning such viruses as polyoma and SV 40, have tended to break down the barrier between oncogenic and lytic viruses (Howatson, 1962), there may be a group of RNA tumour viruses having their own distinctive morphology.

In addition to contrasting the internal structures of influenza and Bittner viruses we were also interested in correlating the negatively stained appearance of Bittner virus with that revealed by thin sectioning. Measurements on negatively stained preparations showed that the internal component, or type A particle, had an average diameter of 750 Å. and the enveloped particle varied within the size range 1000–1500 Å., values that are in good agreement with those obtained by thin sectioning. It is generally accepted that at least some of the type B particles are formed when type A particles move up to, and pass through, the cell membrane. Plate 1, fig. 1, shows a cell border where this is happening, and it can be seen that the internal component of the type B particle is identical with free-lying type A particles in the cytoplasm. The evidence that we present from negative staining shows that particles of a type previously described as type A by negative staining do in fact form the internal component of the more mature particle.

The number of responses to a virus that can be produced by a cell is probably quite limited and hence it is not surprising that a cell membrane with a similar morphological alteration should in one case enclose the type A particles of the mouse mammary tumour and in another case the RNP coil of influenza virus. Similarly, it is not surprising that the widespread viral changes on the surface of the influenza-infected cell would seem to be present also in the Bittner cell surface. For example, when budding particles of the type present in Pl. 1, fig. 2, are seen in negatively stained preparations (Pl. 3, fig. 6), it can be shown that the distinctive projection-covered membrane enclosing the head of the particle also extends the full length of the tail.

The correlation between negatively stained and thinly sectioned immature type B particles of Bittner now seems to be well founded, but the role of the mature type B particle still remains obscure. Negatively stained preparations revealed nothing that would correspond to the mature type B particle of thin sectioning. The only variation seen in the morphology of negatively stained type B particles in which the internal component was visible was a dispersion of the internal body with a

resultant increase in diameter (Pl. 4, fig. 10). Lyons & Moore (1965) suggest that the mature type B particle has a decreased infectivity, and it is possible that the mature type B particle is a degenerate form of the immature type B particle and that when the internal body has lost its integrity it is much more susceptible to the shrinking action of fixatives or dehydrating agents. However, this is merely speculation.

These findings suggest that it may be dangerous to associate viruses too closely on the basis of their external appearance alone. Influenza and the mouse mammary tumour provide an example of two such viruses, as they were found to contain completely dissimilar internal structures. The nucleoprotein component of influenza virus is in the form of a coiled coil approximately 500 Å. in diameter, while the Bittner virus contains a spherical component built up of poorly defined subunits that lack the characteristic pattern of cubic viruses, although this may still be present.

SUMMARY

By the negative staining technique both Bittner and influenza viruses are pleomorphic and have similar fringed surfaces. The present study revealed that Bittner virus had one characteristic form consisting of a head approximately 1000 Å. in diameter and a tail up to 3000 Å. long. On storage at -20° C. Bittner virus broke down to reveal a round internal component of 750 Å. diameter. Influenza virus did not break down on storage at -20° C. but a small number of spontaneously disrupted particles revealed that the internal component was in the form of a coil. The circular internal component of Bittner virus is presumed to be spherical and corresponds to the previously described type A particles as seen by negative staining. The complete enveloped particle corresponds to the type B particle of thin sectioning. It is suggested that the thinly sectioned mature type B particle may be a degenerate form of the so-called immature type B particle. In addition, it is suggested that certain murine RNA tumour viruses may have a morphology distinctive to them.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Part of the border of an acinar cell in a mouse mammary tumour. The cytoplasm contains several doughnut-shaped type A particles, two of which are near to the plasma membrane; in the case of one of them (arrowed) a change can be seen in the cell membrane where the type A particle has made contact with it. Superficial to the cell is another type A particle contained within a cell process and almost completely enrobed in an additional membrane. This enveloped particle would now be referred to as an immature type B particle. × 110,000.

Fig. 2. Another cell edge with several immature type B particles budding from it. At the top and right a number of type A particles can be seen in contact with the cell surface. The extracellular immature type B particles almost all retain a trailer of cell material. Note the constriction at the neck of these particles, a feature present also on the negatively stained particles. (Pl. 3, fig. 6.) × 110,000.

Fig. 3. At the bottom left of this micrograph immature type B particles can be seen budding. Farther out in the lumen of the acinus mature type B particles are present. These are typified by the shrunken nucleoids and less well organized appearance. × 110,000.

PLATE 2

Fig. 4. A group of negatively stained Bittner virus particles from a freshly prepared tumour extract. The particles are pleomorphic and display a distinctive fringed surface. Although there is a suggestion of internal structure in one or two of the particles no definite structure can be seen. × 200,000.

Fig. 5. A group of negatively stained influenza virus particles. As with the Bittner virus particles in Fig. 4 they are fringed, pleomorphic bodies. Like the vast majority of influenza particles none of these particles reveals any internal structure. × 200,000.

PLATE 3

Fig. 6. Although Bittner virus particles are pleomorphic the pattern illustrated here was frequently observed. The particles consist of a well-defined head of approximately 1000 Å. in diameter and a tail which may measure up to 3000 Å. in length. The viral envelope extends to the tail and indicates that the immature type B particles such as are seen in Fig. 2 have virally altered membranes not only around the head of the particles but also extending along the trailer of cell material. Once again these particles are from a freshly prepared extract and no internal component can be seen. × 300,000.

Fig. 7. This micrograph illustrates two features of an influenza virus preparation. The upper part of the figure contains the end of an influenza filament. Like the tail of the Bittner particles in Fig. 6 it is covered by a fringed membrane; unlike Bittner, however, there is no distinctive head at the end. Below this filament is one of the rare influenza virus particles which has spontaneously disrupted and allowed the phosphotungstic acid to delineate the internal structure. This is a hollow strand 60 Å. in diameter wound in a coil approximately 500 Å. in diameter. It is presumed that the strand forming the coil will itself be in the form of a coil or helix. × 300,000.

Fig. 8. The typical appearance of a Bittner particle from a tumour stored for several months at -20° C. About half the particles had changed in permeability and allowed penetration of the PTA. The internal component invariably appeared round as seen here, and is therefore presumed to be spherical in form. The fact that the centre almost always appeared darker makes it seem likely that the sphere is a collapsed one. × 300,000.

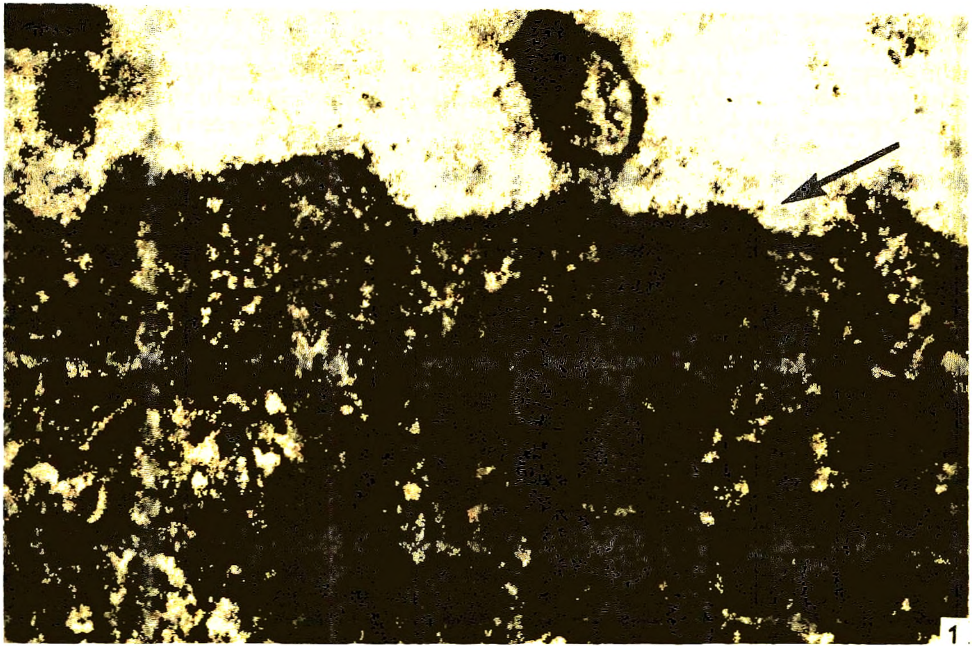
Fig. 9. Occasionally free type A particles were found in the stored preparations. Since these have no outer membrane it is easier to see that these bodies have at places (arrow) a two-layered structure around the periphery and are composed of rather poorly defined subunits. A structure like this, if it had cubic symmetry, would have a hexagonal outline rather than the form seen. × 300,000.

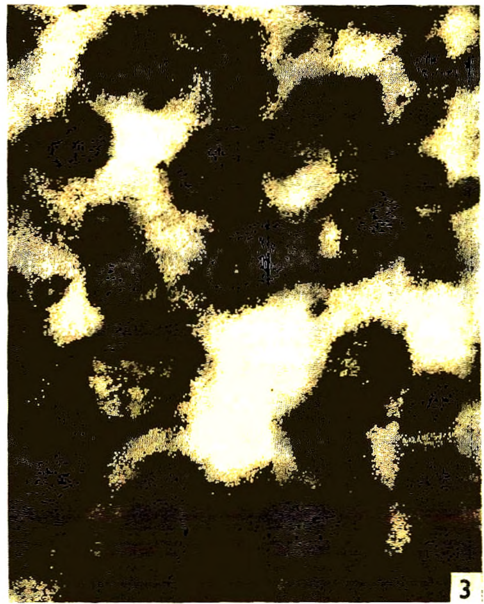
PLATE 4

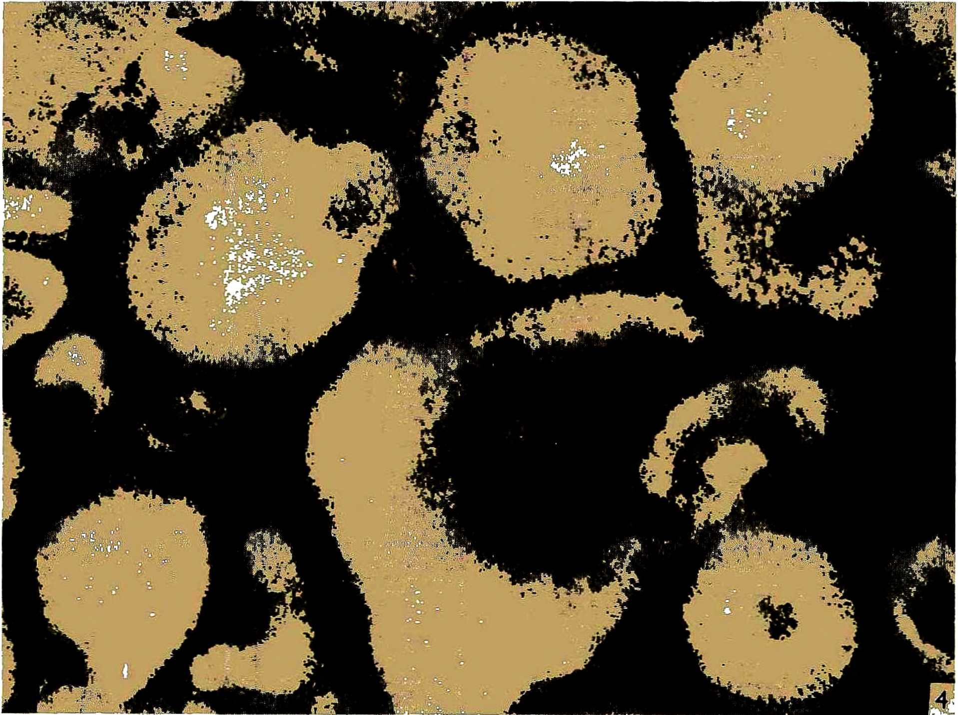
Fig. 10. Of the many PTA-penetrated Bittner particles studied no forms corresponding to the mature type B particle of thin sectioning were found. However, several of the internal bodies did appear to be in a state of disintegration, as is illustrated in the right-hand particle. No further structure was revealed when this occurred and it is suggested that although a disintegrating central body appears larger by negative staining it might shrink on fixation and dehydration and so appear smaller in thin sections. × 300,000.

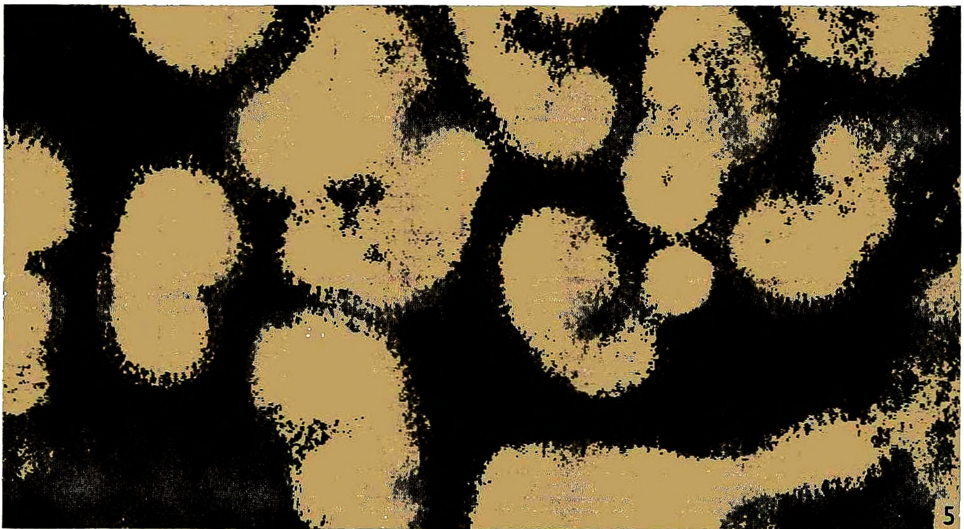
Fig. 11. A Bittner virus particle containing two central bodies. Multiple internal components were found in both Bittner and influenza viruses. × 300,000.

Fig. 12. An influenza virus particle containing several internal components for comparison with fig. 11. × 300,000.









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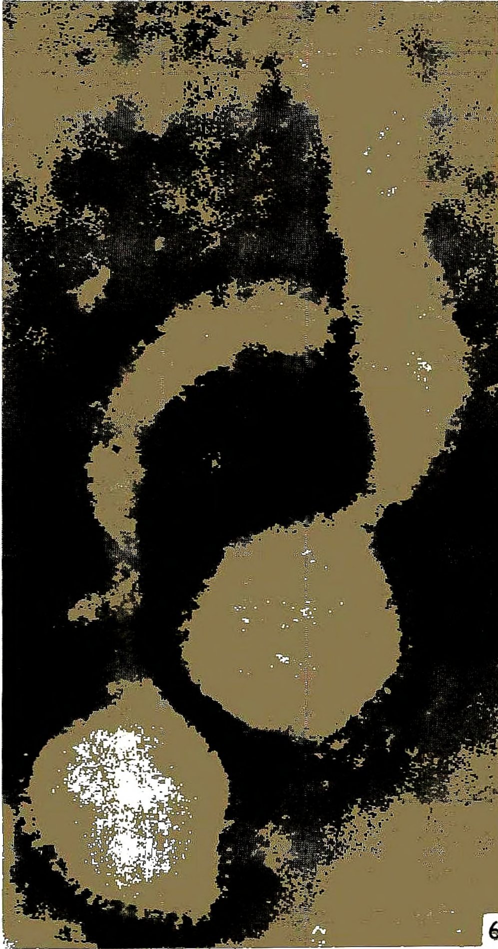
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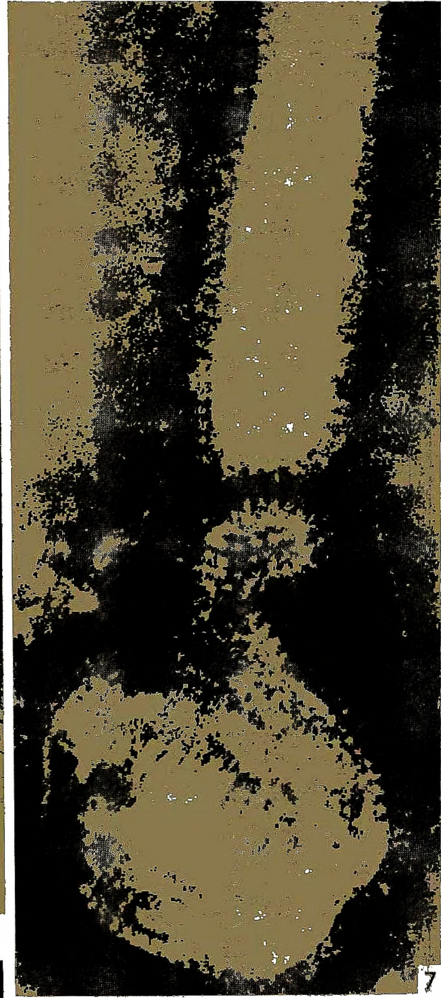
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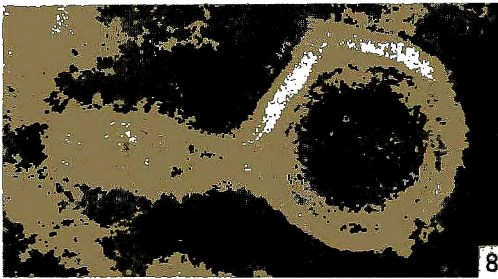
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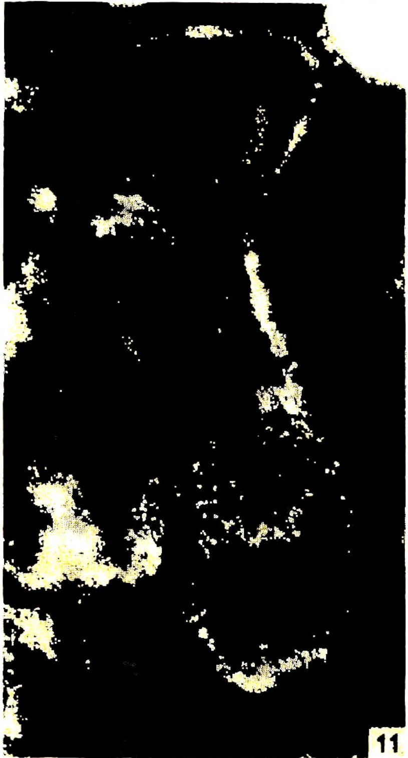
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A study on the virus aetiology of mild respiratory infections in the primary school child

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When a child first attends school he or she immediately joins a group of children highly susceptible to a wide range of infectious agents. The chances of spread of viruses introduced into such a community are very high indeed and each year it can be predicted that outbreaks of the common infectious diseases will almost certainly occur.

The viruses of measles, rubella, chicken-pox and mumps are known to be the aetiological agents of these well-defined clinical illnesses. But besides these there are now known to exist a large number of other viruses which have been found associated with respiratory illness in other populations.

Among the school populations studied by various workers (Bransby, 1951; Norris, 1951; Nisbet, 1956) it seems that children were absent with a respiratory illness between once and twice a year and they lost anything between 2 and 10 days of schooling each year as a result. In a survey of respiratory viral disease McDonald (1963) estimated that among school children respiratory illness accounted for a third of all absence and about three-fifths of time lost.

Since the importance of these new viral agents among the primary school population has not yet been determined a study was undertaken to establish which of these many agents could be found circulating among school children. It was hoped to estimate how much illness was caused by these viruses, how easily they spread and to what extent they were responsible for the loss of school time.

MATERIALS AND METHODS

Population studied

In this country the majority of children attend day schools, and since one of our objects was to observe a population as normal as possible we avoided residential schools where the opportunities for viruses to spread and cause disease are greatly increased, compared with the more usual day school from which young children return to their homes each afternoon.

A local primary school was chosen since it was conveniently near the laboratory and the headmaster was co-operative. The school was recently built, light, airy and warm in winter and mainly served the surrounding housing estate which

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consisted of modern houses for small middle or lower middle class families. Children from all social classes were represented.

Altogether between 265 and 300 children of both sexes and aged between 4½ and 11 years attended the school. They were divided up into eight forms, the first three with the youngest children considered the infants and the rest referred to as the juniors. Class rooms held groups of between 30 and 40 of each age and although there was a common hall for morning assembly and the mid-day meal and a common playground, on the whole each age group tended to keep to itself.

The school year began in September and, with breaks of 2–3 weeks at Christmas and Easter, ended in the middle of July. Permission was obtained from all but a very few of the parents for the children to participate in the study.

Laboratory methods

Visits were made to the school on alternate mornings of the week and any child who was considered by his form teacher, or who considered himself, to be suffering from a cold or other minor respiratory ailment presented himself for examination. Each of us attended in rotation so that differences in clinical criteria and swabbing technique would not influence any long-term trends in the findings. A brief history of the illness was obtained and swabs taken from both the nose and throat. These swabs were broken off into 2 ml. of transport medium consisting of Hanks's salt solution with 0·2% bovine serum albumin. The specimens were taken to the laboratory and inoculated without delay into cultures of HeLa, monkey kidney, human embryo kidney and human diploid lung cells (strain WI-38); blood agar and mycoplasma agar plates were also inoculated. Pools of three specimens were prepared for subcutaneous and intracerebral inoculation into newborn mice. Specimens were then stored in an electric refrigerator at −70° C.

Occasionally cultures of human embryo kidney cells were not available for inoculation on the day the swabs were taken and specimens were then tested after having had one cycle of freezing and thawing.

When inoculated cultures became contaminated or if the cultures themselves were considered unsatisfactory owing, for example, to the presence of latent SV_5 virus in monkey kidney, the specimens were thawed and re-inoculated into fresh cultures.

The production, maintenance and examination of all cultures followed a routine pattern and viruses were identified by standard methods as described in a collaborative study of acute respiratory illness (Report, 1965).

Plates of mycoplasma agar prepared according to the method used by Chanock *et al.* (1962) were inoculated with approximately 0·1 ml. of pooled nose and throat swab extract, and examined at intervals during 18 days incubation aerobically at 37° C. The strains of *Mycoplasma pneumoniae* isolated were identified by fluorescent staining with specific serum.

No attempts were made to obtain blood samples for serology since it was clear that the attendance of children for swabbing would fall sharply if a procedure such as venepuncture were to be suggested. Much of the success of the scheme depended on the willing voluntary co-operation of the children themselves.

The study continued for two consecutive years and besides attempts at virus isolation a record was made throughout this period of the number of children who were absent from school.

RESULTS

Over the 2-year period of the survey swabs were taken from 207 children. In all 782 illnesses were investigated, the numbers in consecutive years being 394 and 388. From these 59 viruses were isolated, 27 in the first year and 32 in the second making a final isolation rate of 8 %.

The distribution of viruses in the various age groups is shown in Table 1. The viruses most frequently isolated were rhinoviruses followed by herpes simplex, respiratory syncytial and influenza A_2 virus. Two strains of a haemadsorbing virus were isolated in monkey kidney cultures from two children in different classes, aged 9 and 11 years, who were swabbed on the same day. These viruses were serologically identical with SV_5 virus. Since the same agents were re-isolated later in human embryo kidney cultures from the original stored specimens, they have been included under the name of para-influenza 5 virus.

Table 1. *Viruses isolated in different age groups*

	Age in (years)						
	4–5	6	7	8	9	10–11	Total
No. of children in age group	50	50	32	30	30	70	262
No. of children swabbed (on one or more occasions)	24	35	29	27	25	67	207
No. of illnesses swabbed	92	142	111	72	151	214	782
	Viruses isolated						
Influenza A_2	0	0	0	1	2	3	6
Para-influenza 1	0	1	1	0	0	2	4
Para-influenza 3	0	1	1	0	1	0	3
Para-influenza 5	0	0	0	0	1	1	2
Respiratory syncytial	2	2	0	0	0	3	7
Herpes simplex	1	3	0	0	1	3	8
Adenovirus	0	0	0	0	0	1	1
Enterovirus	0	1	1	0	0	1	3
Rhinovirus	2	2	7	1	5	6	23
M. pneumoniae	0	0	1	0	0	1	2
Total viruses	5	10	11	2	10	21	59
Percentage of illnesses yielding virus	6	7	10	3	7	10	8

The highest number of viruses was isolated from the 7-year and 10–11-year-old age groups. The variety of viruses was greatest in this latter group where all but para-influenza 3 virus were found.

The number of children investigated in the younger age groups was considerably lower than in the older. The reason for this is not clear; it may be that the younger children were less often suffering from a cold but it is possible that the older children understood the scheme better and attended with more enthusiasm.

Table 2 indicates the seasonal distribution of virus isolations and broadly the myxoviruses were found in the winter months and the picorna viruses (enteroviruses and rhinoviruses) from late spring through to autumn. Herpes simplex was absent through spring and summer except for a single isolation in June.

Table 2. *Monthly virus isolations, November 1962–July 1964*

Virus	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Total
Influenza A_2	.	.	.	.	.	3	2	1	.	.	.	6
Para-influenza 1	.	.	1	1	.	2	.	.	.	.	.	4
Para-influenza 3	.	3	.	.	.	.	.	.	.	.	.	3
Para-influenza 5	.	.	.	.	.	2	.	.	.	.	.	2
Respiratory syncytial	.	.	.	1	4	1	1	.	.	.	.	7
Herpes simplex	.	1	1	1	.	2	2	.	.	1	.	8
Adenovirus	.	.	.	.	.	.	1	.	.	.	.	1
Enterovirus	1	.	1	.	.	1	.	.	.	.	.	3
Rhinovirus	2	1	3	2	1	.	.	2	1	7	4	23
M. pneumoniae	.	.	1	.	1	.	.	.	.	.	.	2
Total	3	5	7	5	6	11	6	3	1	8	4	59

Table 3. *Symptoms in virus positive children*

Virus isolated	No. of of cases	Nasal discharge	Sore throat	Cough
Influenza A_2	6	5	2	1
Para-influenza 1	4	4	1	1
Para-influenza 3	3	3	—	3
Para-influenza 5	2	2	—	—
Respiratory syncytial	7	6	—	2
Herpes simplex	8	8	1	4
Adenovirus	1	1	—	—
Enterovirus	3	3	2	2
Rhinovirus	23	17	5	7
M. pneumoniae	2	1	—	2
Total	59	50	11	22

Mycoplasma pneumoniae was isolated from two cases seen in the winter of 1962–3. Many other strains of mycoplasmas were isolated but as the object was to identify Eaton agent these were not investigated further. *Streptococcus pyogenes* was isolated from two children, one type 4 in January 1963 and the other, type 12, in the following July. This low frequency may be due to the fact that only when β-haemolytic colonies were present in large numbers were they reported as present and investigated further.

Table 3 shows the presenting symptoms associated with the viruses isolated. The most frequent feature was a nasal discharge which was found in 50 cases, nearly half of whom had a cough as well. Only eleven children complained of a sore throat. Four of the six children with influenza A_2 virus infection had no more than mild nasal symptoms as had most of the children with para-influenza 1 virus. In contrast all the three children with para-influenza 3 virus had an associated cough. One of the children with para-influenza 5 virus developed laryngitis and lost his voice. He was absent from school for 2 days as a result.

Most of the infections with respiratory syncytial virus were mild, although two children had a cough and one developed a very bad cold and cough.

Four of the eight cases from whom herpes simplex was isolated had a cough as well as nasal discharge. The rhinovirus infections presented predominantly with nasal discharge, but a third had coughs and a fifth had sore throats.

In both cases where *Mycoplasma pneumoniae* was isolated the main symptom was a persistent cough and it was found possible to re-isolate the organism 1–2 months later.

Table 4. *Virus positive children*

The relation of time absent from school with virus type.

	Days absent					
Virus isolated	None	1	2	3	4	5
Influenza A_2	5	.	1	.	.	.
Para-influenza 1	4	.	.	.	.	.
Para-influenza 3	.	1	2	.	.	.
Para-influenza 5	1	.	1	.	.	.
Respiratory syncytial	5	.	.	1	.	1
Herpes simplex	6	.	1	1	.	.
Adenovirus	1	.	.	.	.	.
Enterovirus	2	1	.	.	.	.
Rhinovirus	21	.	.	.	.	2
M. pneumoniae	2	.	.	.	.	.
Total	47	2	5	2	.	3

Table 5. *Common infectious diseases, 1962–3*

Class	No. of children	Measles	Chicken pox
1	26	17	0
2	38	14	3
3	36	8	0
4	30	0	0
5	39	2	0
6	36	2	0
7	25	0	0
8	35	0	0
Total	265	43	3

Table 4 shows the relationship between school absences, which were determined by examination of the attendance books, and the type of virus in the 59 virus positive children. In 47 (80 %) there was no associated absence from school either before or after the illness. The remaining 12 (20 %) children were absent for varying periods, but none over 5 days. In all three cases where para-influenza 3 was isolated an absence of 1 or 2 days was noted. Para-influenza 1 virus which is often regarded as the more virulent of the two was not associated with school absence in any of the four cases. The longest absences were found in two of the rhinovirus positive children. Neither of the children from whom *Mycoplasma pneumoniae* was isolated was absent from school.

The over-all relationship of respiratory virus isolations with attendance rates is shown in Figs 1 and 2. No significant fall occurred in association with the presence of any of the viruses isolated. The significant alterations are correlated with one

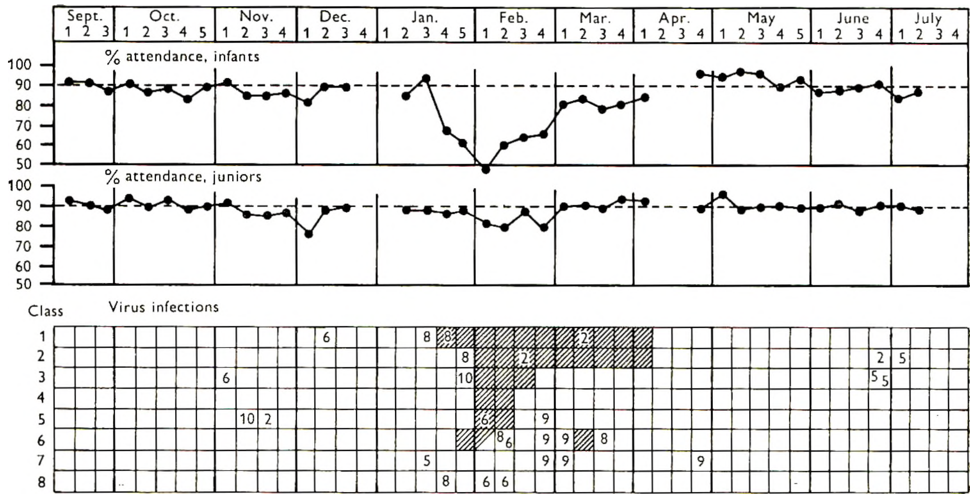

Fig. 1. Courtland School, 1962–3. For key to numbers, see Fig. 2.

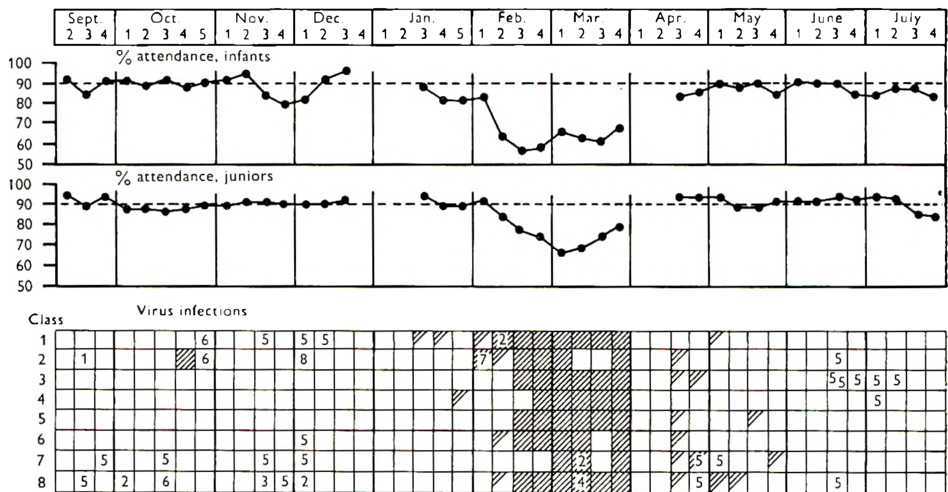

Fig. 2. Courtland School 1963–4

1 = Coxsackie B 3   2 = Herpes simplex   3 = echovirus   4 = adenovirus
5 = rhinovirus   6 = para-influenza   7 = Coxsackie A 9,   8 = Resp. syncytial
9 = influenza A_2 10 = *M. pneumoniae*

or other of the common infectious diseases which are indicated by the shaded areas.

In 1962–3 the sharp fall in attendance in the winter months corresponded exactly with the course of an epidemic of measles which as can be seen in Table 5

affected predominantly the younger age groups. The junior school was barely affected and attendance was maintained at a normal level.

In 1963–4 the winter months were marked by outbreaks of mumps, chicken-pox and rubella. Table 6 shows the occurrence of these diseases in both infants and juniors which reflects clearly in the drop of attendance in all classes, seen in Fig. 2.

Table 6. *Common infectious diseases, 1963–4*

Class	No. of children	Chicken pox	Mumps	Rubella	Measles
1	41	14	12	6	4
2	34	18	1	1	3
3	35	10	3	5	1
4	33	8	5	3	0
5	39	11	6	3	0
6	35	10	6	6	0
7	39	5	2	4	0
8	44	5	5	2	0
Total	300	81	40	30	8

Table 7. *Rhinovirus serotypes*

Date	Age of child (years)	Serotype
1963		
24 Jan.	9	Not identified
27 June	7	NIH 151-1
27 June	7	16/60
4 July	6	Thompson
17 Sept.	10	HIL 181
24 Sept.	9	NIH 151-1
15 Oct.	10	16/60
15 Nov.	10	16/60
22 Nov.	4	Unidentified
24 Nov.	10	Unidentified
3 Dec.	9	B 632
6 Dec.	5	B 632
1964		
28 Apr.	10	NIH 363
28 Apr.	9	16/60
1 May	9	NIH 363
23 June	7	16/60
23 June	10	MRH
23 June	6	Not identified
23 June	7	Not identified
30 June	7	16/60
7 July	7	NIH 151-1
7 July	7	16/60
7 July	8	Not identified

The distribution of the viruses isolated in the course of the study is indicated in Figs. 1 and 2. There is no evidence of any substantial alteration in attendance associated with the presence of any of these agents in the community.

The isolations of influenza A_2 virus coincided with the identification of the virus from cases of clinical influenza, many moderately severe, in other parts of London.

Among the 23 strains of rhinovirus seven different serotypes were identified. Their distribution by time and age is shown in Table 7. The strain 16/60 was found most often followed in frequency by NIH 151-1. Although rhinoviruses appeared to cluster in summer and autumn, the serotypes at any one time were heterogeneous.

DISCUSSION

In this study, if a child became ill at home he would, particularly if he was a young child, quite possibly not attend school and would not be included in this survey. In this way some of the more severe clinical infections with viruses must inevitably have been missed, and it might partly account for the low rate of streptococcal infection. The design of the study meant that on the whole only the mildest infections were sampled.

Perhaps for this reason the number of viruses isolated from these primary school children was low compared with the rate obtained in concurrent studies on children with respiratory illness in hospital or attended at home by their general practitioner, where something like 30% yielded virus (Report 1965). However, all the viruses found associated with the more severe respiratory disease were found circulating at some time or other among the children at school.

There was no evidence of epidemic spread of these viruses and the pattern was one of sporadic infections, of a continuous flow of candidates with colds throughout the whole period, yielding a variety of different associated viruses.

Throughout the survey there was virtually never 100% attendance at school and a remarkably constant figure around 90% was noted except for sharp falls in the early months of each year due to the common infectious diseases.

This pattern could be related to the widespread immunity now known to exist in the pre-school child to many of these newer respiratory viruses which appear to be highly efficient infecting agents even when the children are dispersed in small families. The viruses causing the common infectious diseases appear to be less efficient as spreaders since a high proportion of children reach school age still susceptible to them. It is only when the situation becomes favourable to the virus with multiple non-immunes in a close community that epidemics occur.

The cause of the 10% absenteeism has not been definitely determined. The reason given by parents in the majority of cases where a child is absent for a day or two is a bad cold. From the evidence that is presented here it is clear that there were sufficient viruses circulating in this population throughout the year to account for most of these illnesses and we suggest that they may have been responsible for the low but steady rate of absenteeism observed.

SUMMARY

A survey of mild respiratory disease in a primary school was undertaken over a 2-year period to determine which of the newer respiratory viruses were responsible for these illnesses.

The over-all isolation rate was low but a wide variety of viral agents was isolated.

None of these caused epidemics but it is suggested they may be the cause of a low but constant absentee-rate throughout the year.

We would like to thank the Area Medical Officer of Middlesex County Council Health Department and the Borough Education Officer, Hendon, for making this study possible and the Headmaster and Staff of Courtland School, Mill Hill, for their willing participation. We are grateful to the children and their parents for their co-operation.

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An assessment of oil adjuvant and aqueous influenza vaccines

I. Reactions to the vaccines*

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INTRODUCTION

During the earliest trials of influenza vaccines (Francis & Magill, 1935–6, 1937) reactions to the inoculum were noticed. Hirst, Rickard & Friedewald (1944) found that about 1 in 200 of those inoculated with 1·0 ml. of formalized vaccine developed a fever of more than 100° F. and that moderately sore arms were common. Norwood & Sachs (1947) noted that 77·3 % of a group of industrial workers suffered from sore arms after vaccination and 29·5 % of them became febrile. Salk (1948) showed that reactions were associated with the virus content of the vaccine and not with the presence of impurities. Griffin (1959) found that the proportion of U.S. Army personnel requiring treatment after influenza vaccination fell from 11·33/1000 in 1953 to 2·83/1000 in 1955 and in view of the large numbers of inoculations given this might have reflected an improvement in the vaccine. In Britain, Cope (1960) inoculated over 3000 people and reported 'about half a dozen' were absent from work as a result. Early trials using oil adjuvant vaccines (Henle & Henle, 1945) were marred by the high incidence of local reactions yielding residual sterile abcesses, and Beebe, Simon & Vivona (1964) showed that cysts occurred in 3 % of subjects in large-scale trials of 1951–3 although an improved technique of inoculation was used. However, using a purified emulsifying agent, Himmelweit (1960) found none of these reactions in his group of 160 volunteers.

Although it seemed from these recent trials that both the aqueous and oil adjuvant vaccines were now relatively innocuous, Clarke (1962) and also Meichen, Rogan & Howell (1962) reported that contemporary commercial aqueous influenza vaccines were causing an undesirable number of reactions. Because of these conflicting reports, trials were carried out to assay reactions to various types and doses of influenza vaccines and this paper describes the results.

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MATERIALS AND METHODS

Trials

Two trials were carried out, the first over the winter of 1962–3 and the second in 1963–4.

First trial

In the first trial there were two parts. In both parts the volunteers were first to fifth-year medical students at the Queen's University of Belfast. In the first part, which began in November 1962, all volunteers whose date of birth was an odd number (e.g. 1st, 3rd, etc. of the month) were given aqueous vaccine, and those with even-numbered birthdays received placebo. The second part started in January 1963, in such a way that there were 8 weeks between inoculations. The same group of volunteers took part, with a few changes detailed below, and now all those with birthdays in the first half of a month were given an adjuvant vaccine, and those born in the second half of a month received aqueous vaccine.

In each part inoculation was carried out in the morning and the volunteers were examined on the same afternoon, and thereafter on the 1st, 2nd, 3rd and 7th days, and finally 21 days after the first inoculation and 28 days after the second. Blood samples for antibody studies were taken at the time of inoculation and at each last examination.

At every examination each volunteer's card was marked with the degree (0–3) of local or general reactions found. The card bore columns for erythema, pain, tenderness, lymphadenitis, malaise, fever, generalized aches and pains, nausea or vomiting, diarrhoea or constipation, headache, coryza, allergic manifestations and other signs and symptoms. Neither the volunteers nor the examiners were aware of which inoculum had been given until after the trial.

Second trial

In the second trial the volunteers were first-year medical students of the Queen's University or nurses from the Royal Victoria and Royal Maternity Hospitals, Belfast. All these volunteers were given a new adjuvant vaccine and were examined 2, 7, 28 and 90 days after inoculations. The same manifestations were sought as in the first trial, but the layout of the cards was modified and improved. Owing to the absence of a control group, the information can only be regarded as supplementing that from the first trial. Blood samples were taken at inoculation and at 28 and 90 days afterwards.

Vaccines

The first inoculations of the 1962–3 trial were 0·5 ml. volumes of either placebo (phosphate-buffered saline) or aqueous vaccine (*Invirin*, Glaxo). These were administered subcutaneously over the triceps. In the second part of the trial either 1·0 ml. *Invirin*, subcutaneously, or 0·25 ml. oil adjuvant vaccine, into the belly of the triceps, was given. In the second trial the new, augmented, adjuvant vaccine (*Admune*, Evans Medical) was inoculated into the upper part of the long head of the triceps.

Analysis

In this paper statistical significance was assessed using the χ^2 test without Yates's correction and significance was read when $P \leqslant 0{\cdot}05$. Where the expected number was too small to use χ^2, an exact probability was calculated.

Table 1. *The nature of inocula used in the trials, with their antigenic content in haemagglutinating units (HAU).*

	Trial 1962–3				
	First part		Second part		Trial 1963–4
Strains of influenza virus	Placebo	*Invirin* (aqueous vaccine)	*Invirin* (aqueous vaccine)	Adjuvant vaccine	*Admune* (adjuvant vaccine)
A/Singapore/1/57	—	3750*	7500	1500	1500
A/England/1/61	—	1250	2500	500	500
B/England/939/59	—	2500	5000	1000	1000
B/Taiwan/4/62	—	—	—	—	500
	Phosphate buffered saline 0·5 ml.	In 0·5 ml. dose	In 1·0 ml. dose	Per 0·25 ml. dose	Per 0·25 ml. dose

* Haemagglutinating units.

RESULTS

First trial, part 1 (*November–December* 1962)

Three hundred and eighty volunteers took part, but only 281 were examined on every occasion and yielded complete records. The remainder missed one or more sessions. Absenteeism was apparently not governed by the vaccine received ($0{\cdot}3 > P > 0{\cdot}2$), but was associated with the inconvenience of attending for examination. The results of those who had incomplete records were not markedly different from those with complete records. The figures supplied refer to the records of all volunteers.

Local reactions

The principal local reactions were erythema, pain and tenderness.

Erythema around the site was scored from 0 to 3. An area up to 15 mm. in diameter was indicated by '1', more than 15 mm. by '2', and widespread erythema by '3'.

Pain was assessed by asking volunteers whether their arms hurt or hindered them and was scored '0', '½' (minimal), '1' (slight), '2' (moderate) and '3' (severe). Minimal pain amounted to awareness of having been inoculated.

Tenderness was elicited by the response to questioning and firm palpation of the inoculated area. It was graded in the same way as pain.

Compared with the placebo injection of 0·5 ml. of saline, the same volume of *Invirin* produced reactions significantly more often. Erythema only occurred after

the vaccine and there were more reports of tenderness from those who were given vaccine at each examination for the first 3 days ($0{\cdot}05 > P > 0{\cdot}025$ some hours after inoculation, $P < 0{\cdot}0005$ on days 1–3). However, a few hours after the inoculation 'pain', as defined above, appeared to be a function of the injection process itself because it was only on the second examination that the vaccinated group yielded more complaints, ($0{\cdot}01 > P > 0{\cdot}005$).

Table 2. *The incidence of the main local reactions associated with the first trial, part 1*

Reaction		Placebo 0·5 ml., 186* (%)	*Invirin* 0·5 ml., 194* (%)
Erythema	0	100·0	68·5
	1	0	13·5
	0	0	17·6
Pain	0	81·8	72·4
	½	7·5	13·5
	1	10·2	12·4
	2	0·5	1·7
Tenderness	0	93·0	58·5
	½	3·2	20·7
	1	3·2	19·2
	2	0·6	1·6

* Number of volunteers.

Although induration and swelling of the inoculated arm only occurred in those who received vaccine the incidence was small, the greatest on any day being 6/175. An attempt to use the measurement of arms as an index of reaction had to be abandoned as swelling was so inconspicuous. Both types of inoculation caused bruising at the site in a proportion of cases, but rather more bruises followed vaccine. The greatest number was seen on the third day when 10·3 % of the vaccinated group and 6·0 % of those with placebo had bruises.

Systemic reactions

There was no evidence that this small dose of vaccine caused systemic reactions. In the first 3 days after the inoculation, coryza, influenzal symptoms and malaise were more common among those given placebo (coryza in 3·8 % against 1·5 %). Two cases of migraine and one of paroxysmal tachycardia during the week after inoculation occurred in students given placebo.

No allergic manifestations occurred before 3 days after inoculation. After this, one case of a transient maculo-papular rash and one of localized urticaria occurred in students who had been given vaccine and a more pronounced urticaria in one of those given placebo.

General responses

On the seventh day, each volunteer was asked whether the inoculation had interfered with work, play or social activities. Five complaints were received, three from volunteers who had received placebo and two from those given vaccine.

First trial, part 2 (January–February 1963)

Three hundred and forty-two volunteers took part in this phase, and 267 were examined on all occasions. Three extra volunteers who had received no vaccine before joined the trial; 26 of the placebo group and 15 who had had vaccine left the trial. The reactions of those who left had been no more severe than the average.

Table 3. *The incidence of the main local reactions associated with the first trial, part 2*

Reaction		Adjuvant vaccine, 158* (%)	*Invirin* 184* (%)
Erythema	0	99·4	87·5
	1	0	5·4
	2	0·6	7·1
Pain	0	53·8	44·6
	½	24·7	26·1
	1	19·0	23·9
	2	1·9	5·4
Tenderness	0	36·1	22·3
	½	39·9	43·5
	1	20·2	28·8
	2	3·8	5·4

* Number of volunteers.

Local reactions

Erythema, pain and tenderness were analysed in the same way as previously.

The dose of *Invirin* recommended by the manufacturers is 1·0 ml. and this contains five times as much antigen as a dose of adjuvant vaccine. However, the presence of the oily vehicle and the intramuscular site of deposition of the adjuvant vaccine might be expected to offset the reduction in reactions caused by the smaller amount of antigen.

The adjuvant vaccine was not associated with erythema and the full, 1·0 ml., dose of *Invirin* caused less reddening of the skin than the half-dose given previously. About half those inoculated complained of pain and the arms of two-thirds or more were tender. *Invirin* elicited complaints of both pain and tenderness from a greater proportion of the volunteers than adjuvant vaccine and in both instances this excess is found in the early examinations. *Invirin* in general was associated with a larger incidence of reactions and caused them more rapidly but for rather a shorter time. By 7 days, however, there were no complaints of pain but 10 of tenderness (3 %) from all the volunteers.

As in the first part of the trial induration and swelling were only found in a few

volunteers (four and two, respectively) and all occurred following *Invirin*. Bruising at the inoculation site, however, occurred more frequently with the adjuvant vaccine (eight cases) than with *Invirin* (four). This was possibly because of the different mode of inoculation. One student suffered transient symptoms in the distribution of the radial nerve after intramuscular injection of adjuvant vaccine.

No chronic swellings, induration or cystic lesions referable to the adjuvant were found either in those who had received this vaccine a month after inoculation or in any of the 143 of 158 volunteers of this group examined 8 months later.

Systemic reactions

It was difficult to detect any unequivocal pattern of these. There were 12 reports of coryzal symptoms among those given *Invirin*, and seven after adjuvant vaccine ($0 \cdot 4 > P > 0 \cdot 3$). In addition, one case of migraine, two of headache and two of malaise occurring within 3 days of inoculation were all within the group given *Invirin*. However, as this disparity of cases of coryza and other constitutional symptoms was also present at 7 and 28 days after inoculation the relation of these symptoms to the vaccines is not clear.

None of the volunteers said that the inoculation had interfered with work, play or social activities.

Table 4. *The replies to the question, 'Was the inoculation worth while?' at the end of each part of trial* 1

	Trial 1			
	First part		Second part	
Answers	Placebo 0·5 ml., 186* (%)	*Invirin* 0·5 ml., 194* (%)	Adjuvant vaccine 0·25 ml., 158* (%)	*Invirin* 1·0 ml., 184* (%)
'Yes	80·0	84·5	67·7	67·9
'No'	3·8	4·6	8·2	11·4
'Don't know'	10·2	7·7	10·8	9·8
No answer recorded	6·0	3·1	13·3	10·9

* Numbers of volunteers in each group.

General responses

In both parts of the trial, at the time of the final examination, 3 and 4 weeks after the inoculation respectively, the volunteers were asked whether they thought the inoculation would be worth while if it prevented influenza. In neither part of the trial did the answers distinguish between the different inocula, but there was a significant difference between the responses to the first and second parts of the study ($0 \cdot 025 > P > 0 \cdot 01$).

Those volunteers who had been in both parts of the trial were asked how the two inoculations received compared. No significant preference was revealed for one vaccine over another ($0 \cdot 7 > P > 0 \cdot 6$) or for placebo over vaccine.

Second trial (November 1963–February 1964)

There were 97 volunteers in this trial of whom 29 were nurses.

Local reactions

The incidence of reactions associated with the use of adjuvant vaccine in the first trial and *Admune* in the second were broadly similar regarding erythema

Table 5. *The distribution of replies when volunteers who had participated in both parts of the trial were asked, 'How did the second inoculation compare with that of the first part of the trial?' (trial* 1)

	First inoculation 0·5 ml. placebo. Second part inocula		First inoculation 0·5 ml. *Invirin*. Second part inocula	
Answers	Adjuvant vaccine 0·25 ml., 73* (%)	*Invirin* 1·0 ml., 88* (%)	Adjuvant vaccine 0·25 ml., 83* (%)	*Invirin* 1·0 ml., 95* (%)
'Better'	28·8	20·45	28·9	33·7
'Worse'	39·7	44·3	36·1	35·8
'Don't know'	12·3	14·8	18·1	18·9
No recorded answer	19·2	20·45	16·9	11·6

* Number of volunteers in each group.

Table 6. *The incidence of the main local reactions associated with the second trial compared with the corresponding data from the first trial*

		Second day		Seventh day	
Reaction		*Admune* adjuvant vaccine, second trial, 97* (%)	Adjuvant vaccine, first trial, 143* (%)	*Admune* adjuvant vaccine, second trial, 96* (%)	Adjuvant vaccine, first trial, 154* (%)
Erythema	0	95·9	99·3	100·0	100·0
	1	3·1	—	—	—
	2	1·0	0·7	—	—
Pain	0	63·9	76·0	99·0	100·0
	½	20·6	12·7	—	—
	1	14·4	10·6	1·0	—
	2	1·0	0·7	—	—
Tenderness	0	56·7	53·5	96·9	98·7
	½	23·7	33·1	1·0	1·3
	1	15·5	9·9	2·1	—
	2	4·1	3·5	—	—
Bruising	0	92·7	94·4	92·7	98·1
	½	2·1	2·1	1·0	0·6
	1	8·2	2·8	5·2	1·3
	2	4·1	0·7	1·0	—

* Number of volunteers in each group.

($P = 0{\cdot}16$), pain ($0{\cdot}2 > P > 0{\cdot}1$) and tenderness ($0{\cdot}2 > P > 0{\cdot}1$). However, in the second trial more complaints of pain were recorded from the nurses than from the students.

Bruising was much more marked in the second trial, and, in the main, this was accounted for by the high incidence among the nurses. This might have been associated with the use of larger gauge needles for inoculating this group.

Transient swelling of the inoculated arm was reported by three nurses and three medical students.

Miscellaneous complaints included one, from a nurse, of mild regional axillary lymphadenitis for 2 days after inoculation. At 28 days another nurse reported some residual stiffness in the inoculated arm. On examination no objective sign was found.

In three of the nurses and in one student, all women, subcutaneous nodules at the site of inoculation were detected at the examination at 28 days. These nodules were fairly well defined and slightly tender on pressure. They were separate from the skin and underlying muscle and first appeared about 2 weeks after inoculation. On follow-up, one had disappeared 2 months after inoculation. In the other three, the nodules regressed slowly, and by 6 months were barely detectable.

Systemic reactions

No allergic manifestations were reported or seen. Inoculation was followed by a feeling of faintness in one case, and a nurse reported that a 'cold' started on the evening of the inoculation. Two volunteers complained of attacks of nausea and vomiting starting 24–36 h. after the inoculation. In one case, these symptoms also occurred in unvaccinated home contacts.

General responses

Of 96 volunteers questioned on the 7th day, nine said that the inoculation had troubled or hindered them. Eight of these were nurses. All graded the nuisance as 'slight'. Although none of the volunteers felt that the inoculation had curtailed work, play or social activities, two of the male students said they would not be inoculated again—because they never got influenza!

DISCUSSION

The evidence above shows that standard doses of aqueous and adjuvanted influenza vaccine caused a substantial number of local reactions. If those persons who had only minimal pain and tenderness are discounted, 1·0 ml. of *Invirin* caused about the same incidence of local reactions as recorded by Clarke (1962). The adjuvant vaccine, in comparison, caused rather fewer reactions; a finding which agrees with Howell & Mackenzie (1964).

On the other hand, although Clarke (1962) found 30 % had general malaise and 6 % allergic reactions and Howell & Mackenzie (1964) reported that influenza-like symptoms soon after injection and malaise persisting for a fortnight occurred in 9·8 and 22·4 % of those given adjuvant and 8·5 and 22·4 % of those given

aqueous vaccine respectively, in our experience systemic reactions were not a problem.

The authors of many reports have found it difficult to assess the acceptability of vaccines, particularly against less dreaded diseases, in objective terms. In the current trials the incidence of pain and tenderness revealed objectively did not correspond with the paucity of those who said the inoculations curtailed activities. However, the fact that nurses, doing more manual work, were more affected than students and the increased proportion of students denying the 'worthwhile'-ness of vaccine after a second inoculation tend to reinforce doubts (Howell & Stott, 1964, Richardson & Kilpatrick, 1964) whether an average population would support a programme of annual re-inoculation.

In addition, as children are particularly prone to reactions (Davenport & Hennessy, 1960), much less irritant vaccines would be needed to attempt the logical step of controlling influenza epidemics by vaccinating schoolchildren. The purified haemagglutinin vaccine described by Davenport *et al.* (1964) could well provide a more acceptable product.

The oil adjuvant vaccine presented a problem of its own. The occurrence of persistent nodules in some of our volunteers and an incidence of cysts of 3·3/10,000 in the Medical Research Council (1964) trial contrast with the earlier, more optimistic, reports (Himmelweit, 1960; Meiklejohn, 1962). For vaccination that is liable to be repeated and particularly for a disease of the nature of influenza such local reactions are not acceptable (Tyrrell, 1966). Also, with so small a dose of antigen much of the pain and tenderness associated with the inoculation was probably due to the adjuvant components themselves. McCarthy (1964), using hexadecane and octadecane, has demonstrated the ability of mammals to metabolize preparations of straight-chain hydrocarbons given orally. However, from the observed persistence of oil at the site of adjuvant inoculations this breakdown must occur slowly in the tissues and possibly the equivalent fatty acids that are formed during metabolism may themselves be irritant just as the greater toxicity of early batches of adjuvant was due to excess free oleic acid in the emulsifying agent.

Several approaches to solve this difficulty have been made. Wilner *et al.* (1963), in limited experiments, found that a pure branched-chain hydrocarbon oil produced a better adjuvant effect than the usual Drakeol 6VR with less toxicity. Workers at the Merck Institute (Woodhour *et al.* 1964; Peck *et al.* 1964) have reported on the efficacy of a metabolizable adjuvant comprising peanut oil, aluminium monostearate and Arlacel A and found in animal experiments that the reactions produced were quantitatively less than with mineral oil. Herbert (1965) described a 'multiple emulsion' in which the adjuvant emulsion was dispersed in fine droplets in an aqueous phase and thus might be less able to cause cysts. It is likely that one, or a combination of these developments, may lead to more acceptable adjuvant vaccines.

While the vaccines used in the trials did not cause many systemic reactions they are clearly not entirely satisfactory but there is evidence that better products may be forthcoming.

SUMMARY

Trials of aqueous and oil adjuvant vaccines in young adult volunteers showed that severe local reactions were rare. However, the incidence of minor symptoms was too high for a vaccine which requires to be administered repeatedly. In contradistinction to some reports, systemic and allergic reactions did not constitute a problem.

I should like to thank my colleagues at the Department of Microbiology for their assistance in inoculating, bleeding and checking reactions in the volunteers. Without the co-operation of the volunteers this study would not have been possible. I also wish to thank Prof. G. W. A. Dick for his encouragement and advice, and Mr T. D. Merrett, who kindly gave advice on the statistical analysis of the results. The vaccines and placebo were supplied by Glaxo Laboratories Ltd., and financial assistance for the study came from the Northern Ireland Hospitals Authority and the National Fund for Research into Poliomyelitis and other Crippling Diseases.

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An assessment of oil adjuvant and aqueous influenza vaccines

II. Antibody responses to the vaccines*

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(*Received* 19 *May* 1967)

INTRODUCTION

Over the winters of 1962–3 and 1963–4 trials were carried out to ascertain the reactions to contemporary aqueous and adjuvant vaccines which contained strains of both influenza A and influenza B (Forsyth, 1967). It was felt that an assay of the antigenic efficacy of these vaccines was an essential part of this investigation. In addition, several of the recent trials of British-made influenza vaccines, evaluated in terms of haemagglutinating units (HAU), were done with special material with only one antigenic variant (M.R.C. 1955, 1957, 1958; Himmelweit, 1960, Hobson, *et al.* 1964) and it seemed that further information relevant to commercially available vaccines could be gained by serological tests on the volunteers.

This paper describes the results of these tests.

MATERIALS AND METHODS

Trials and vaccines

The vaccines used and the nature and organization of the trials have been described in the previous paper (Forsyth, 1967).

Virus strains

An avid strain of A/Singapore/1/57 was kindly supplied by Dr A. S. Beare (Colindale), and Dr D. Hobson (Evans Medical Ltd.) sent B/England/939/59. This latter was the most avid of a number of antigenically similar strains.

Antibody titrations

Haemagglutination-inhibition (H.I.) tests were performed in a standard manner using plastic plates, 0·2 ml. volumes and 8 HAU of virus antigen. The test plates were incubated at 4° C. and the agglutination patterns read and scored in the usual manner.

All sera were inactivated with cholera filtrate (N. V. Philips-Duphar) and made up to a 1/10 dilution with 0·01 M phosphate-buffered saline, pH 7·0.

* This material was included in a thesis submitted to the University of Capetown in partial fulfilment of the requirements for the degree of Doctor of Medicine.

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Neutralization tests were carried out and the results read as described by Beare (1962). The same strains of virus were used as in the H.I. tests.

In all series compared, sera from any one person were tested together against each antigen.

RESULTS

First trial

During the period of the first trial no evidence of widespread influenza in the community was received by the Virus Reference Laboratory nor was there any significant change in the levels of antibody of those who were only inoculated with saline placebo.

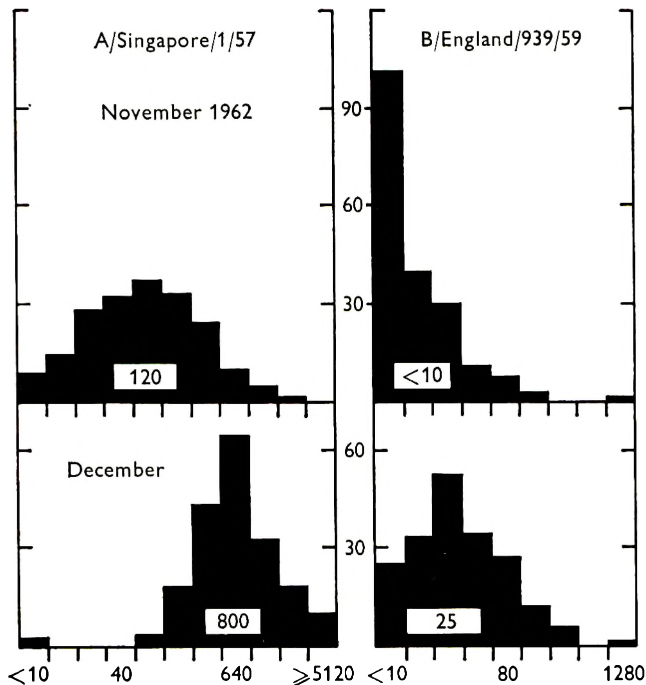

Fig. 1. The effect of an inoculation of 0·5 ml. *Invirin* on H.I. antibodies. Median titres inserted in each histogram.

One hundred and ninety volunteers were given 0·5 ml. of *Invirin*. This was one-half the dose of vaccine recommended by the manufacturer.

Before inoculation there was a striking difference in the distribution of titres of antibody to the two principal antigens. Very few persons (8) lacked detectable antibody to A/Singapore/1/57, but the majority (101) did not have antibody to B/England/939/59. Three weeks after inoculation even this small dose of vaccine had stimulated high levels of antibody to the influenza A strain. Twenty-five per cent of those without antibody to A/Singapore/1/57 or B/England/939/59 failed to convert serologically. However, in the case of influenza B not only were the antibody titres elicited poor, less than 25 % being 80 or more, but the numbers of subjects left without detectable antibody were large.

From individuals in the second part of the trial, sera taken before the second inoculation and 4 weeks and 9 months after inoculation were tested together. Therefore, the 1·0 ml. *Invirin* (aqueous vaccine) and the adjuvant vaccine were compared in, effectively, two populations; one without previous vaccine experience and the other having had the 0·5 ml. dose of *Invirin* previously.

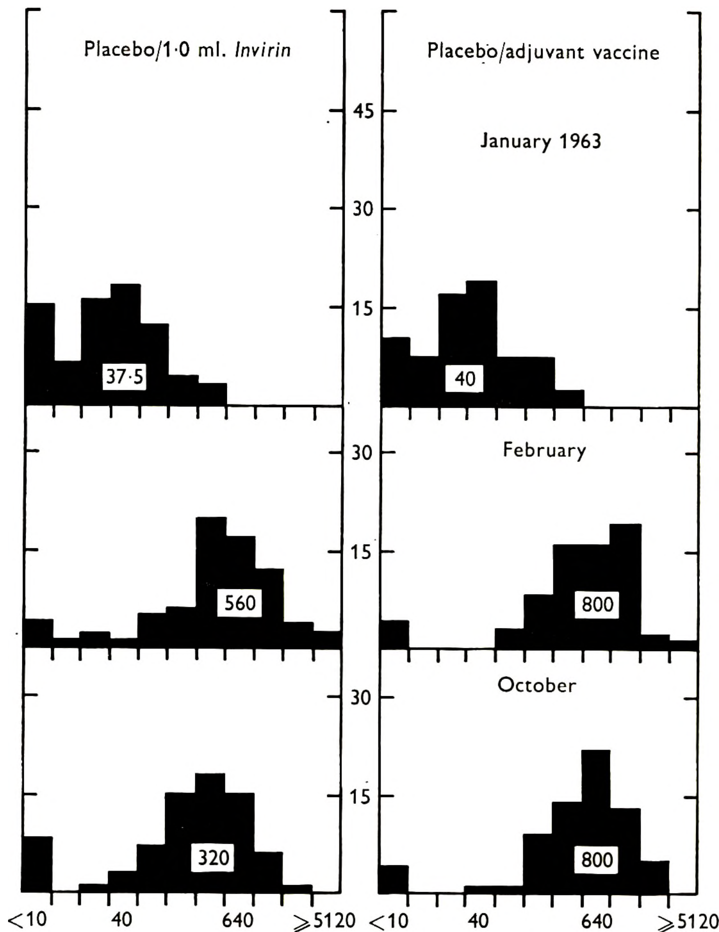

Fig. 2. The distribution of antibodies to A/Singapore/1/57 before, and at intervals after the inoculation of either 1·0 ml. *Invirin* or adjuvant vaccine, in subjects previously given saline placebo.

In those who had had no vaccine previously

Antibody to influenza A. The 1·0 ml. dose of *Invirin* given to 74 volunteers was associated with a rise of the median antibody titre from 37·5 before inoculation to 560 a month later. Once again, about a quarter (4 of 15) of those without antibody before failed to acquire it afterwards. However, 9 months after inoculation the median antibody level had fallen to 320 and now eight people showed no antibody.

Following the adjuvant vaccine, the median titre of the 69 volunteers rose from 40 to 800. While the distribution of titres associated with the two vaccines was

not significantly different at 1 month ($0{\cdot}2 > P > 0{\cdot}7$), at 9 months the advantage of the adjuvant vaccine was clear ($0{\cdot}01 > P > 0{\cdot}005$). Although the adjuvant vaccine failed to stimulate antibody in four of the 10 volunteers without it, after 9 months no others lacked detectable antibody.

Antibody to influenza B. For this antigen the aqueous vaccine, *Invirin*, gave a better over-all result than the adjuvant vaccine. The former not only caused a

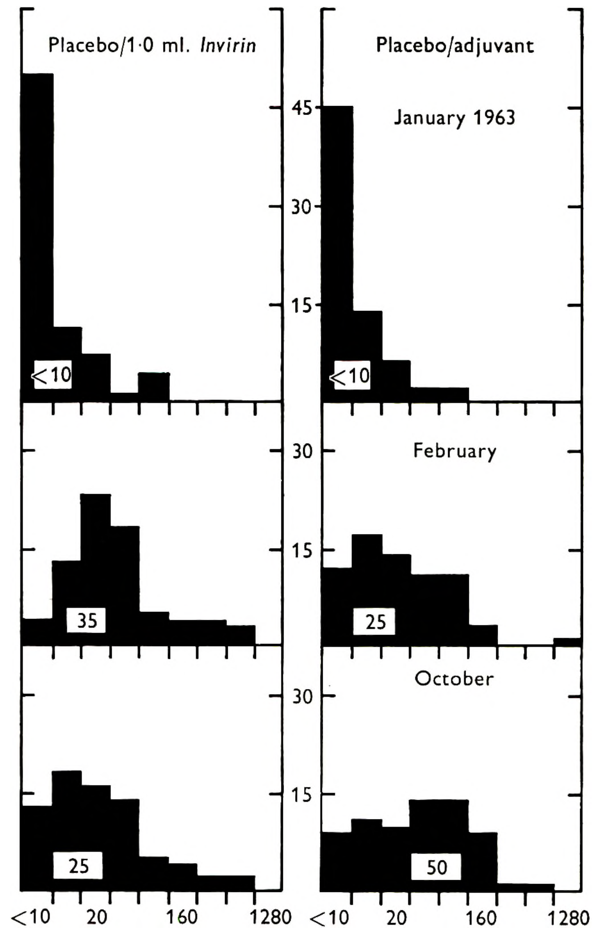

Fig. 3. The distribution of antibodies to B/England/939/59 before and after the administration of *Invirin* or adjuvant vaccine to subjects previously given saline placebo

significantly ($0{\cdot}005 > P > 0{\cdot}001$) better antibody response at 1 month but also elicited antibody in a greater proportion of those previously negative (47 of 51, as against 33 of 45). Nine months after inoculation the adjuvant vaccine showed to advantage but the differences in antibody titre between the groups were not significant ($0{\cdot}2 > P > 0{\cdot}1$).

In those who had had 0·5 *ml.* Invirin *previously*

Antibody to influenza A. A further dose of aqueous vaccine caused virtually no alteration in antibody level a month later and at 9 months the median titre was the same as before the inoculation and was no better than the group which had received a single 1·0 ml. dose of *Invirin.* These results were much inferior ($P <$ 0·0005) to those after adjuvant vaccine both at 1 month and 9 months. Adjuvant vaccine caused a considerable rise in median titre—from 320 to 800—and this was essentially maintained 9 months after immunization.

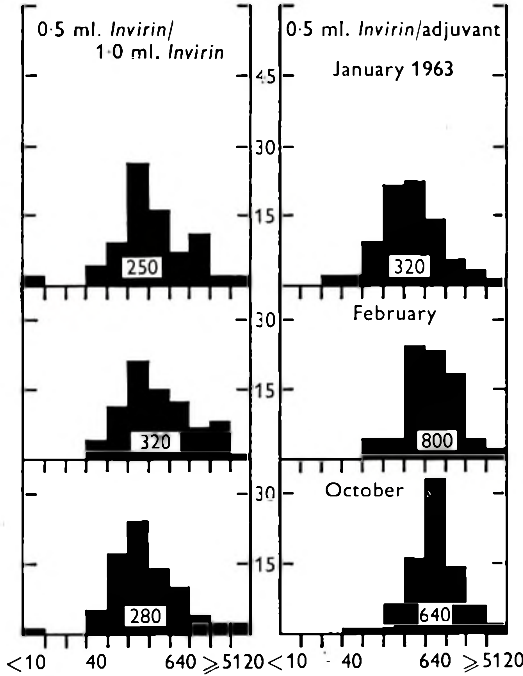

Fig. 4

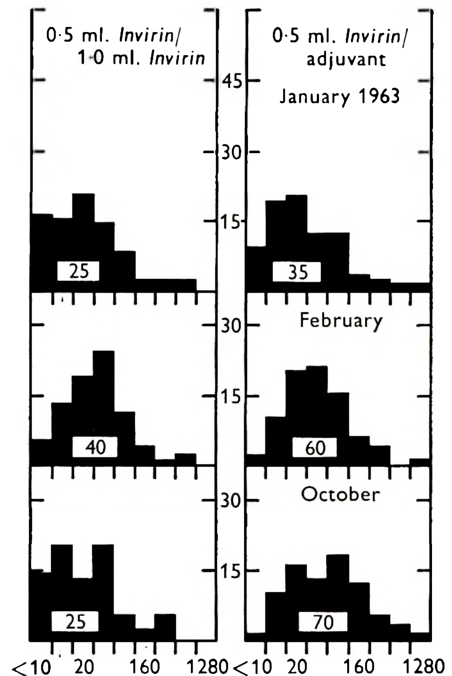

Fig. 5

Fig. 4. The distribution of antibodies to A/Singapore/1/57 before and after the administration of *Invirin* or adjuvant vaccine to subjects previously given 0·5 ml. *Invirin.*

Fig. 5. The distribution of antibodies to B/England/939/59 before and after the inoculation of either 1·0 ml. *Invirin* or adjuvant vaccine in subjects previously given 0·5 ml. *Invirin.*

Antibody to influenza B. In contrast to the results in the volunteers who received placebo for the first injection (see Fig. 3), the adjuvant vaccine here gave better results than *Invirin.* While the titres achieved at a month were no better ($0·7 > P > 0·6$), at 9 months the difference was marked ($P <$ 0·0005). At the same time, of the 16 who had no detectable antibody before 1·0 ml. of *Invirin,* 14 lacked antibody at 9 months but after the adjuvant only one of nine failed to have antibody at this time.

Second trial

In the second trial (1963–4) 97 volunteers were given the adjuvant vaccine, *Admune*. They were bled at the time of inoculation and 1 and 3 months later. Once more there was no evidence of widespread influenza in the community

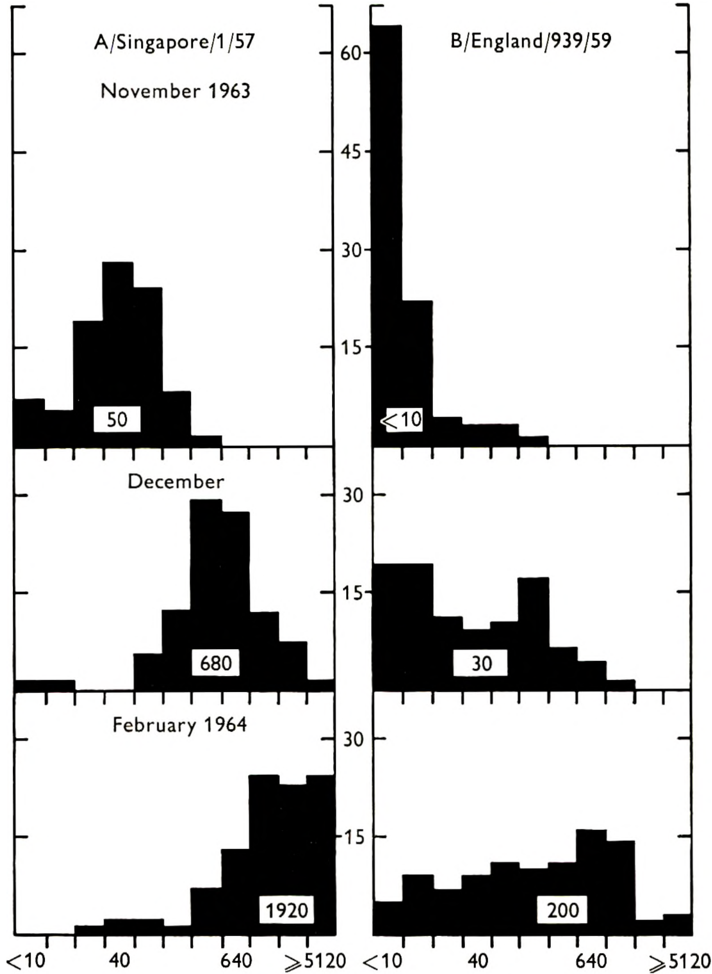

Fig. 6. The distribution of antibodies to two strains of influenza before and at intervals after a single inoculation of *Admune* adjuvant vaccine.

during the period of the trial. The new group of volunteers exhibited the same low incidence of antibody to influenza B as noted in the previous trial.

Antibody to influenza A. Four weeks after inoculation the antibody titres had risen to the levels similar to those achieved in the earlier trial. At 3 months there had been a further substantial rise to a median titre of 1920. As only a small sample of those in the first trial had been bled at a similar interval no direct comparison can be made.

Antibody to Influenza B. Here again the results at 4 weeks were similar to those

receiving adjuvant vaccine in the first trial, both in regard to median titre and in the proportion failing to acquire antibody. At 3 months, however, the median titre had risen to 200 and now only five out of the initial 64 remained without antibody.

Neutralization tests

The low levels of H.I. antibody to influenza B could have been an artifact caused by poor avidity of the virus strain used. This possibility was investigated by performing neutralization tests on a sample of 419 sera from the first trial. The same strain of virus was used as in the H.I. tests.

Of 137 that showed no antibody (< 10) with H.I., with the neutralization test, 97 gave the same result, 12 showed no antibody at the 20 level and 28 showed antibody. In 26 sera found to be positive by H.I., no antibody was shown by the neutralization test.

DISCUSSION

Salk & Laurent (1952) showed that influenza virus antigen in a water-in-oil emulsion with oily adjuvant elicited higher and more persistent levels of antibody than antigen in aqueous suspension. Also, after the adjuvant vaccine, antibody continued increasing a month after inoculation. Hobson *et al.* (1964) disputed this last finding but it is confirmed in the present study.

With influenza A the adjuvant vaccine tested gave results equal to those of the aqueous vaccine, *Invirin*, at a month and far superior at 9 months despite containing only a fifth as much antigen. It has been shown that individuals appear to have a 'ceiling' level of antibody to influenza not affected by further antigenic stimulation (McDonald & Andrews, 1955). This is shown here with *Invirin* but the adjuvant vaccine seems to be able to overcome this, possibly to establish a 'ceiling' at a higher level (Hobson *et al.* 1964).

Low initial levels of antibody to influenza B were a constant feature in the volunteers tested and this was, in the main, confirmed by the neutralization tests. Thus, despite the prevalence of influenza B in Northern Ireland in 1961–2 (Forsyth, 1962) it seems that the vaccine strains represented a primary antigenic stimulus for many of the volunteers. This explains the poor response to influenza B. It is consistent with previous reports on the poor antibody response which occurs against new antigenic variants of influenza virus (Meiklejohn & Bruyn, 1949; Appleby, Himmelweit & Stuart-Harris, 1951; McCarroll & Kilbourne, 1958).

In contrast to the findings with influenza A neither the half dose of *Invirin* nor the adjuvant vaccine gave as good a response of influenza B antibody as the 1·0 ml. dose of *Invirin*. Even in the second trial with *Admune* the median response of antibody to B/England/939/59 was no better a month after inoculation than with the earlier adjuvant vaccine. However, although the amount of influenza B antigen in *Admune* had been increased substantially this was by means of the relatively dissimilar B/Taiwan/4/62 variant.

The poor effect of adjuvant relative to aqueous vaccine at 1 month can be attributed to a delayed response by those volunteers lacking pre-inoculation antibody. On analysis it is seen that the antibody levels elicited by the two vaccines

were similar in volunteers with antibody before inoculation ($0{\cdot}99 > P > 0{\cdot}975$) but different in those without ($0{\cdot}01 > P > 0{\cdot}005$). This difference had disappeared at 9 months. The delayed response did not occur in people given *Invirin* previously.

Throughout these trials the experience with antibody to B/England/939/59 confirms the overwhelming importance of previous experience with the antigen. None of the vaccines used provided high average titres rapidly in people without preinoculation antibody.

SUMMARY

In trials with polyvalent commercial influenza vaccines the antibody responses to oil-adjuvant vaccine persisted longer and were often higher. Antibody conversion was poor after all vaccines and delayed after adjuvant.

We wish to thank Drs A. S. Beare, D. Hobson and H. G. Pereira for supplying strains of virus and for their advice on technical problems.

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A yellow fever vaccine free from avian leucosis viruses

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The virus of yellow fever is present in large areas of tropical South America and Africa, where it circulates amongst the animal and human populations with occasional outbreaks of overt human disease, the epidemiology of which is not yet fully understood. There is, accordingly, likely to be need for an indefinite period for a protective vaccine both for travellers, as required by the International Sanitary Regulations, and, when needed, for the containment of epidemics. The well-known attenuated 17 D strain of yellow fever virus is now almost universally used for vaccination and is in fact the only strain recognised by W.H.O. for use under the above Regulations. This was produced over 30 years ago by passage of the wild Asibi strain of yellow fever virus, first in mouse embryo tissue and then a large number of times in chick embryo tissue cultures, resulting in marked attenuation of the viscerotropic and, particularly, of the neurotropic properties (Theiler, 1951). A number of substrains were produced and all those in use today are of the 200th to 300th subculture in chick embryo tissue cultures or in whole chick embryos, the latter still remaining the most suitable material in which to grow the virus for vaccine production.

Because of the possible oncogenic potential in mammalian tissue of known and unknown viruses of the avian leucosis complex (Vogt, 1965), opinion is growing that these viruses should be excluded from human vaccines to be given parenterally, and this is now a requirement for measles vaccines made in chick embryo tissue (W.H.O., 1966*a*). Because they are widespread in many flocks of chickens, and no special precautions were taken to exclude them, it is probable that most substrains of 17 D yellow fever virus are contaminated with avian leucosis viruses. Harris *et al.* (1966) found heavy contamination in a batch of secondary seed used for vaccine production in these laboratories, and suggested that the standards for yellow fever vaccine should conform to those for other vaccines made in chick tissues, and this opinion has also been voiced by a W.H.O. scientific group (W.H.O., 1966*b*). Piraino, Krumbiegel & Wisniewski (1967) describe the discovery of leucosis viruses in several different batches of yellow fever vaccine in the U.S.A. Like Harris *et al.* (1966) they were unable to detect neutralizing antibodies in human recipients of such vaccines, but both these teams of workers used only the Bryan standard strain of Rous sarcoma virus (see below under Methods) as the challenge virus in serum neutralization tests and, as the former comment, may have missed antibodies formed against other antigenic groups.

In any manipulations needed to clear a 17 D vaccine substrain of leucosis viruses it is important that these should not involve more than a few passages of the virus

beyond the level at which it has been found to be both safe and effective as a vaccine, because of the risk of inducing undesirable variations in its properties. In the early days vaccines were commonly made by serial passage of virus from one batch of chick embryos to another; on one occasion, in Brazil, several batches of vaccine made at passage levels 22 to 25 away from a substrain of known safety were found to have increased neurotropic properties, causing encephalitis, mostly not fatal, in up to 2% of those vaccinated, all ages being affected. This was correlated with an increased neurovirulence on intracerebral inoculation into rhesus monkeys (Fox, Lennette, Manso & Aguiar, 1942; Fox & Penna, 1943). Conversely, on another occasion, some further passages beyond the 300th possibly resulted in greater attenuation with a reduction in immunizing efficiency below acceptable levels (Soper & Smith, 1938). Although in several other instances repeated passages of several substrains have not resulted in any changes (Theiler, 1951), the proven risk that these may sometimes occur led to the introduction of the well-known 'seed-lot system', now routinely applied to the production of all virus vaccines, whereby vaccine is always made at a fixed passage level. Following the introduction of this system cases of encephalitis and other undesirable reactions after vaccination with the 17 D yellow fever virus have been rare and sporadic. In an account of a recent fatal case of encephalitis in a 3-year-old child (Sencer *et al.* 1966) it was estimated that about 100 million doses of the vaccine must have been given since 1942, when the 'seed-lot system' was started, but only 15 other cases of encephalitis have been reported in the world literature in this time, all in infants under a year old and none of them fatal (Stuart, 1956; Feitel, Watson & Cochran, 1960). It is unknown whether such cases are due to the occasional emergence of virus particles with increased neurotropic properties, by analogy with the happenings with attenuated poliomyelitis virus (see, for example, Permanent Section of Microbiological Standardization, 1967), or due to an unusual susceptibility of the infant nervous system to invasion by the virus, coupled, perhaps, with an anomalous antigen-antibody reaction of the type suggested by Webb & Smith (1966). The fact remains that 17 D yellow fever is a potentially neurotropic virus and must be treated as such. Most countries now do not demand the vaccination of infants under a year old.

This paper describes the methods used to free a 17 D substrain from contaminant leucosis viruses, with the minimum number of passages, and the production and testing of seeds and vaccine derived from it.

METHODS AND MATERIALS

The presence of leucosis viruses was tested for by the interference method (RIF test) originally devised by Rubin (1960). The yellow fever virus in the material for test was neutralized with serum from rabbits hyperimmunized with the French neurotropic strain of virus, propagated in mouse brains. The material was then inoculated on primary tissue cultures of chick embryos from a flock of chickens shown to be free from leucosis viruses and with a uniform sensitivity to Rous sarcoma viruses. The cultures were serially passaged and at the 4th subculture

both the test and negative control cultures were challenged, some with the Bryan standard strain of Rous sarcoma virus (B-RSV), of antigenic subgroup A, and others with a Rous sarcoma virus pseudotype (RSV-RAV 2) of antigenic subgroup B. Occasionally, challenges were done at the 2nd subculture. The test was considered negative if the test cultures showed a less than tenfold reduction in foci as compared with the negative control cultures when these were counted 7 days after the challenge.

The production of vaccine seeds and of final vaccine was done by methods in routine use in these laboratories. Six- to 7-day-old chick embryos were inoculated directly through the shell with 0·1 ml. of virus suspension and were incubated for 3·5–4 days at 37·5° C. The surviving embryos were then harvested aseptically, in groups of 20, and fragmented mechanically in a stainless steel blender, using 40 ml. of sterile distilled water for each group. This suspension was clarified by light centrifugation and the supernatant fluid, after taking samples for virus titration and bacterial sterility tests, was stored in glass containers at −70° C. After completion of these tests, sufficient sterile batches were taken to make a convenient lot, they were thawed rapidly, pooled, and equal volumes filled into ampoules and freeze-dried. The chick embryos were taken from pathogen-free flocks of chickens, established by these laboratories from accredited sources, which are held in isolated quarters and repeatedly tested to confirm their freedom from leucosis viruses and other adventitious agents. Both seeds and vaccine were tested according to the W.H.O. Requirements for Yellow Fever Vaccine (1959) some of which are considered in detail later.

Titrations of yellow fever virus were done by inoculating 0·03 ml. of fourfold dilutions intracerebrally (i.c.) into groups of five to six 1-month old Swiss mice, bred in these laboratories. Some titrations were also done in monkey kidney tissue by an interference method (Draper, to be published) of almost equivalent sensitivity. Virus titres and doses are given in terms of mouse LD 50 doses (MLD 50). Neutralization (NT) tests were done by incubating, at 37° C. for 60 min., mixtures of equal amounts of test sera, previously heated at 56° C. for 30 min., and of serial tenfold dilutions of the French neurotropic strain of yellow fever virus (in its 541st passage), and these were then inoculated i.c. into groups of mice as above. The neutralization index (N.I.) of a postinoculation serum is the difference between the $-\log_{10}$ LD 50 titres of the virus in it and in the preinoculation serum. For haemagglutination-inhibition (HI) tests sera were absorbed with kaolin and then goose erythrocytes before use. Twofold dilutions starting from 1/20 were incubated at 4° C. overnight with 4 to 8 haemagglutinating units (HAU) of a yellow fever virus antigen, prepared by high-speed centrifugation (11,000 *g*) of an aqueous suspension of mouse brains infected with the French neurotropic virus, before the addition of goose erythrocytes at a pH of 6·6.

RESULTS

Production of leucosis-free seeds and vaccines

A batch of vaccine, number 1815, manufactured in 1945 by the Rockefeller Foundation Laboratories, New York, was received by these Laboratories in 1946. This material represents the 230th passage of the yellow fever 17D substrain (M. Theiler, pers. comm., 1965) and has been used as primary seed for the production of several batches of secondary seed, from which large amounts of vaccine have been made, amounting to several million doses. It was subjected to RIF tests, as described, on several occasions and found to be heavily contaminated with leucosis agents, resistance of the cultures to both of the challenge viruses being evident. Some preliminary experiments showed that it was possible to clear the material of leucosis viruses by limit dilution passages through chick embryo tissue cultures, combined with treatment with a leucosis antiserum, and this was also achieved by passage through human embryo fibroblast cultures. However, as six or more passages were needed and poor virus yields were obtained at the end, another attempt was made in whole chick embryo with a more potent antiserum, hoping thereby to reduce the number of passages and to obtain a higher yield of virus. Serial tenfold dilutions of vaccine 1815 were incubated at 37° C. for 60 min. with a chicken antiserum against the RPL12 virus strain of leucosis virus, and these mixtures were then inoculated into batches of leucosis-free chick embryos. Harvested wet material was stored at − 70° C. while RIF tests and tests for yellow fever virus content and for bacterial sterility were done. On conclusion of these, one batch, made from 16 embryos which had been inoculated with a 1/1000 dilution of vaccine 1815 with antiserum, giving a dose per embryo of about 10^2 MLD50 of 17D virus, was chosen as being suitable for a new primary seed, in that it was found to be free from leucosis viruses and contained a good yield of 17D yellow fever virus. Some of this material was diluted 1/1000 and was inoculated by itself into a larger batch of chick embryos, each of which received again about 10^2 MLD50 of 17D virus. The wet pulps harvested from 117 embryos were stored in batches at − 70° C. while a pooled sample from each was subjected to a RIF test and 17D virus titration, and bacterial sterility tests were done on the individual batches. All these tests proved satisfactory and the material was then further diluted with water and a large batch of ampoules freeze-dried. This was designated as a new leucosis-free secondary seed (YFS/5) to be used for the production of vaccine. The primary seed material was also freeze-dried in ampoules, for future use, and both it and YFS/5 satisfied criteria for sterility, guinea-pig toxicity, and residual moisture as defined by W.H.O. (1959), while the identity of the 17D yellow fever virus in both was confirmed by NT tests with a specific antiserum. Furthermore, quantities of YFS/5 equivalent to over 5000 egg inoculation doses were tested in primary monkey kidney tissue cultures, in chick embryo fibroblast and liver cultures, by inoculation into the allantoic cavity and the yolk sac of chick embryos, and by inoculation i.c. and intraperitoneally into mice, for the presence of other extraneous viruses, all with negative results. For these tests the 17D virus was neutralized with the rabbit antiserum described. Using a

dilution of YFS/5 such as to give an inoculum per egg of about $10^{2\cdot5}$ MLD50, over 30 batches of 17D vaccine have been made in pathogen-free eggs; all have been found free of leucosis viruses in the test employing two different challenge viruses. Such vaccine represents the 233rd passage level of the 17D strain.

Table 1. *Results of inoculation into Macacus rhesus monkeys of yellow fever secondary seed virus*

No. of monkey	Wt. in Kg.	Inoculum	No. occasions when viraemia detected (see text)	Highest dilution of serum in which virus detected	Days post inoculation when temp. exceeded 40 °C.	Days post inoculation when some paralysis (see text)	Maximal paralysis seen in limbs (see text)	Days post inoculation when maximal paralysis	Clinical assessment encephalitis (see text)	Neutralization index of post inoculation serum
2	7·2		Nil	Nil	5, 10–12	Nil	Nil	Nil	0	3·8
3	6·0		2	Undil.	10–13	14–18	5545	14–18	1 +	2·4
4	6·2		1	1/10	14–15	14–29	5533	16–18	2 +	2·1
5	5·1	$10^{4\cdot9}$	Nil	Nil	Nil	12–19	5533	14	1 +	3·6
6	6·0	MLD50	1	Undil.	8–9, 11–13	12–18	5544	12–18	1 +	3·6
9	6·9	i.c. of	3	1/10	8–9, 12	12–32	5533	14–22	3 +	4·2
11	5·8	YFS/4/2	1	1/10	10–15	12–28	5545	15–28	1 +	2·8
14	6·2	(standard	2	Undil.	8–13, 15	14–18	5554	14–18	1 +	1·5
15	4·9	seed)	3	Undil.	10–12	12–28	5533	14–15	3 +	1·9
16	5·1		1	Undil.	10–12	14–21	5544	14–18	1 +	2·8
30	4·1		2	Undil.	10–11	14–24	5554	14–24	1 +	3·3
33	5·0		2	Undil.	9–11	12–29	5534	14–19	3 +	3·0
1011	5·0		1	Undil.	8–11	10–13	5545	10–13	1 +	1·9
1012	2·8	$10^{3\cdot9}$	3	1/10	5–6, 8–11	13	5554	13	±	1·8
1013	3·0	MLD 50	3	1/10	13	6	5544	6	±	2·2
1014	3·1	i.c. of	1	Undil.	8–10	11–19	5544	11–13	1 +	3·0
1015	3·5	YFS/5	2	Undil.	8–10	12–15	5544	12	1 +	2·2
1021	3·8	(leucosis-	3	1/10	6, 10–11	11–24	5534	14	1+	3·3
1024	3·9	free seed)	Nil	Nil	6, 10–12	11–20	5522	13	3 +	2·4
1024	4·6		2	Undil.	9–10, 12	11–18	5534	15	1 +	3·2

Tests of viscerotropism and neurotropism

The W.H.O. Requirements for Yellow Fever Vaccine (1959) state that each new batch of seed shall be subjected to these tests in not less than 10 monkeys, and Table 1 shows the results obtained in individual monkeys with two different batches of secondary seed. The first, YFS/4/2, was a new batch of standard secondary seed made directly from vaccine 1815, and the second, YFS/5, was the new leucosis-free secondary seed, the production of which has just been described. *Macacus rhesus* monkeys, which had previously been found to be free of antibodies for arboviruses of antigenic group B, were each inoculated, under anaesthesia, into a cerebral hemisphere with 0·25 ml. of a suspension of the seed for test and this was immediately afterwards titrated to determine the virus content. The requirements state that each monkey shall receive a dose of not less than 5000 MLD50. The monkeys had rectal temperatures taken daily, and on the 2nd, 4th, and 6th days after inoculation venous blood samples were taken. The serum was rapidly separated from these and at once inoculated i.c. into groups of mice, undiluted and in tenfold dilutions up to 10^{-3}, in order to assay the degree of viraemia. The mice were observed for 3 weeks and the brains were harvested from any dying with signs of yellow fever encephalitis. The presence of the virus was confirmed in several mice from each test by its neutralization by specific antiserum. For detecting any signs of encephalitis the monkeys were removed from their cages each day over a period of a month and placed one at a time in a special exercise cage, where a full

range of movements involving running, jumping, and climbing could be seen. The same pair of observers recorded their observations throughout, and in the rare case of disagreement the verdict went in favour of recording the greater amount of impairment of movement. The scale used for grading the amount of weakness of the limbs follows that of Bodian (1948), the individual limbs being listed in this order: right arm, left arm, right leg, left leg. The figure 5 denotes normal; 4 minimal weakness; 3 marked weakness, but able to use the limb; 2 denotes inability to use the limb for climbing but able to lift it against gravity; 1 denotes inability to lift the limb against gravity; 0 denotes complete flaccid paralysis. A clinical assessment was also made of the overall picture, following that of Nathanson *et al.* (1966*a*, *b*) in which: 0 = normal; ± = doubtful (clumsy, or tremors for 1 or 2 days); 1+ = minimal (clumsy or slow, in grades 4 or 5 for 3 or more days, or tremors for 3–10 days); 2+ = moderate (grade 3 paralysis in at least one limb for 2 or more days but without residual paralysis, or tremors for 11 or more days); 3+ = severe (grade 3 or lower in at least one limb for 2 or more days with definite residual weakness after a month); 4+ = fatal. Thirty days after inoculation blood samples were again taken from the monkeys and the sera tested for neutralizing antibodies.

Table 2. *Summary of tests in monkeys of yellow fever vaccine secondary seeds*

	Standard YFS/4/2	Leucosis-free YFS/5
No. of monkeys with viraemia on all 3 days tested	2	3
No. of monkeys with viraemia on 2 days only	2	4
No. of monkeys with viraemia on 1 day only	4	2
No. of monkeys without demonstrable viraemia	2	1
Total no. of positive sera in which virus detected only when undiluted	11	16
Total no. of positive sera in which virus detected when diluted 1/10	3	3
Total no. of positive sera in which virus detected when diluted 1/100	0	0
No. of monkeys with assessment of encephalitis as 3+	2	2
No. of monkeys with assessment of encephalitis as 2+	1	0
No. of monkeys with assessment of encephalitis as 1+	6	6
No. of monkeys with assessment of encephalitis as ±	0	2
No. of monkeys with assessment of encephalitis as 0	1	0
Average number of days per monkey when temperature exceeded 40° C.	3·6	3·2
Mean N.I. of postinoculation sera	2·8	2·6

The data in Table 1 are summarized in Table 2. Regarding the degree and duration of viraemia both seeds appear to be similar though the viraemia is a little more in the case of YFS/5, perhaps related to the smaller size of the monkeys available for this test. In this test YFS/4/2 fails the criterion of the W.H.O. requirements which demands that virus shall be isolated from at least 9 out of 10 monkeys, but, as both the monkeys without demonstrable viraemia (nos. 2 and 5) subsequently became fully immune, this must have occurred at some stage. The other criteria are met that 'in no case shall 0·03 ml. of serum contain more than 500 MLD50 and in not more than one case shall 0·03 ml. of serum contain more

than 100 MLD 50'. Regarding the important criterion of neurotropism, the W.H.O. requirements state that 'not more than 20% of the test monkeys shall develop encephalitis manifested by paralysis or inability to stand, with or without subsequent death'. If a 3 + grade of paralysis or higher as defined above, is taken as indicating such obvious clinical encephalitis both batches of seed can be deemed to fulfil the criterion. It is probable that most of the monkeys with a 1 + or even with a 2 + paralysis would not have been detected without the use of an exercise cage. There is no evidence of a change in neurotropism with the YFS/5 seed, while the febrile reaction, which has been roughly correlated with the neurotropic properties (Fox & Penna, 1943), is similar with the two seeds. All the monkeys became immune.

Table 3. *Average survival times* (A.S.T.) *of mice inoculated with varying doses of* 17D *yellow fever vaccines derived from two different secondary seeds*

$\log_{10}$ dose of virus in MLD 50	From standard seed YFS/3		From leucosis-free seed YFS/5	
	No. mice	A.S.T.	No. mice	A.S.T.
0·1–0·5	400	12·5	136	12·8
0·6–1·0	377	11·4	169	11·7
1·1–1·5	372	10·7	131	11·6
1·6–2·0	14	9·9	49	9·9

Neurotropism in mice

Average survival times (A.S.T.) have been calculated for mice inoculated intracerebrally with batches of standard vaccine derived from a secondary seed YFS/3, a sister seed of YFS 4/2, or with leucosis-free vaccine derived from YFS/5. For the calculations, only mice dying of typical yellow fever encephalitis have been included and, contrary to the occasional practice of allotting an arbitrary figure for survival time for mice remaining alive at the end of the observation period of 3 weeks, these have been omitted altogether. This follows the method of Meers (1959) in his study of the adaptation of the 17D virus to mouse brain. Table 3 shows the A.S.T.'s for 30 batches each of the two different kinds of vaccines according to the dose inoculated. As these have been calculated from routine titrations of vaccines, designed to cover the end-points, there are few observations with the larger doses. There are no differences in the neurovirulence for mice, and the figures are similar to those given by Meers (1959) for unadapted 17D virus.

Tests of antigenicity in man

As a final and necessary comparison, two comparable groups of young adults, all previously free from antibodies to group B arboviruses, were inoculated subcutaneously, one with standard vaccine and the other with leucosis-free vaccine. Several different batches of each type of vaccine were used, and in each case titration of remaining ampoules showed that from $10^{4 \cdot 1}$ to $10^{4 \cdot 4}$ MLD50 were given. Samples of venous blood were taken at the time of inoculation and again after 1 month and these were tested for the development of neutralizing and HI

antibodies. The results are given in Table 4; there are no differences in the responses obtained. One subject in each of the two series had failed to develop detectable antibodies at the time that the second sample of blood was taken, by the conventional tests used here, and it is possible that these would have been found in a later sample. Smith, Turner & Armitage (1962) and Smith, McMahon & Turner (1963) found that in a few yellow fever vaccinees antibodies may not appear until after 28 days.

Table 4. *Neutralizing and HI antibodies in sera of human subjects inoculated with different* 17D *yellow fever vaccines*

Vaccine	No. inoculated	Mean N.I.	G.M.T. HI antibodies
Standard	38	1·6	70
Leucosis-free	59	1·7	80

DISCUSSION

The steps taken to free a substrain of 17D yellow fever virus from contaminant leucosis viruses have not caused any changes in its other properties. This, perhaps, was to be expected in view of the simplicity of the manipulations, in that it was achieved with the minimum number of passages and without recourse to other measures, such as limit dilution, which might favour the chance emergence of mutant particles. Nevertheless, because of the widespread use of this vaccine, the possibility had to be excluded.

The methods in present use for assessing in particular the neurotropic properties of a vaccine substrain of the virus are crude when compared with those used, for example, in testing attenuated strains of poliomyelitis viruses (Permanent commision of microbiological standardization, 1967). Although the 17D virus is obviously neurotropic when introduced directly into the nervous system, and although, at least in the case of some substrains, it is not in a stable state of attenuation, the very large number of vaccinations done, with remarkably few undesirable reactions, testify to the safety of its use. However, in consonance with modern trends towards improved vaccines, it might be at least reassuring if more critical tests could be adopted. A move in this direction has been made by Nathanson *et al.* (1966*a*, *b*) who have developed a feasible method for assessing the neurovirulence of arboviruses in monkeys by the examination of specific 'indicator centres' in the brain and spinal cord.

SUMMARY

1. The methods used for clearing a vaccine substrain of 17D yellow fever virus of contaminant avian leucosis viruses are described.

2. Tests in animals and human subjects showed that the characteristics of the virus remained unchanged.

The RIF tests and the preparation of the leucosis antiserum were done by Messrs A. E. Churchill and W. Baxendale, while the monkey neurovirulence tests

were done in collaboration with Dr V. Udall, all of these laboratories, whose help is gratefully acknowledged. Major-General M. H. P. Sayers and Colonel L. G. Irvine R.A.M.C. kindly arranged for the antigenicity tests in human subjects. Mr A. Jopling gave much valuable technical assistance throughout.

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Ward floors and other surfaces as reservoirs of hospital infection

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INTRODUCTION

The floors of hospital wards become contaminated with large numbers of bacteria, including *Staphylococcus aureus*, and are commonly assumed to be important reservoirs of hospital infection. To prevent the dispersal of bacteria from floors into the air, various improvements in methods of cleaning have been introduced, notably oiling of floors, the use of oiled mops and, with most success, the use of special vacuum cleaners (van den Ende, Lush & Edward, 1940; Bate, 1961; Babb, Lilly & Lowbury, 1963). Efforts are also commonly made to reduce the numbers of bacteria on the floors by manual or mechanical scrubbing or disinfection, but the results of such treatment have been disappointingly small (Finegold *et al.* 1962; Vesley & Michaelson,1964). Ayliffe, Collins & Lowbury (1966) found that areas of floor protected against recontamination lost about 80 % of their bacterial flora after mopping or mechanical scrubbing, and a significantly larger proportion (about 99 %) after treatment with certain disinfectants. Since areas which were not protected against recontamination were often as heavily contaminated 1 hr. after scrubbing or disinfection as they were before such treatment, there appeared to be little or no advantage in cleaning floors. On the other hand, frequent scrubbing or the use of disinfectants might be expected to keep the mean level of bacterial contamination lower that that which is present on an uncleaned surface. Even if regular disinfection of floors reduces the mean level of contamination, such treatment cannot be considered useful in preventing infection unless pathogens on the floor are transferred either by air or by contact to patients in the ward.

In this paper we describe studies on the equilibrium levels of floor contamination and the influence of various factors, including disinfection, on these levels. We also describe experiments on the redispersal by air movements of settled dust containing *Staph. aureus* and discuss floor bacteria as a source of infection in the light of the results obtained. Studies on the bacterial contamination of walls and on the use of tacky and antiseptic mats are also described and discussed.

GENERAL METHODS

Contamination of surfaces by a disperser

A carrier and profuse disperser of *Staph. aureus* co-operated in experiments on contamination of surfaces by shedding these organisms into the environment during exercise and by direct transfer from fingers. This subject is subsequently referred to in the paper as 'the disperser'. The staphylococcus isolated from the disperser was not typable by phages at the routine test dilution (R.T.D.) but when tested at 1000 R.T.D. it was found to be of type 80/81; it was sensitive to penicillin and resistant to tetracycline, novobiocin and neomycin (see also Ayliffe & Collins, 1967).

Bacteriological methods

Nutrient agar containing phenolphthalein diphosphate (Barber & Kuper, 1951) was used for settle plates, slit-sampling plates, and impression plates from surfaces (Foster, 1960). Total counts and counts of presumptive *Staph. aureus* were made after incubation for 18 hr. at 37° C. A selection of colonies of presumptive *Staph. aureus* isolated in each experiment was subcultured on blood agar and subsequently confirmed by slide or tube coagulase tests. Strains of *Staph. aureus* were tested for sensitivity to a range of antibiotics by a ditch plate method.

EXPERIMENTAL STUDIES

THE ACCUMULATION OF BACTERIA ON WARD FLOORS AND SURFACES

In a previous study (Ayliffe *et al.* 1966) it was found that floors of surgical wards became contaminated rapidly after cleaning or disinfection. The contamination was probably due both to airborne bacteria and to contact with shoes and trolley wheels. As there was no obvious increase in bacterial floor counts between 1 and 9 hr. after cleaning, it seemed that a 'plateau' may have been reached in which organisms were being removed at about the same rate as they were being deposited. Such a plateau phenomenon has been observed in studies on the contamination of stainless steel surfaces in clean rooms examined over a period of many weeks (Michaelson, personal communication; Favero *et al.* 1966).

In this study, the relative numbers of bacteria deposited on a ward floor from the air and by contact were assessed; the accumulation of bacteria on ward floors and on initially clean squares of vinyl left exposed and unwashed in the same wards was studied for periods up to 4 weeks.

Sources of contamination of floors

Methods

An area of floor in a surgical ward was cleaned with a disinfectant or detergent, and part of the treated floor was immediately covered with a cardboard box open on the underside (Ayliffe *et al.* 1966). Two settle-plates were exposed on top of the box for 1 hr. One hour after treatment of the floor, two impression plates were taken from the covered area and two from the uncovered area of floor. Ten experiments were performed.

Results

Table 1 shows the mean bacterial counts from covered and uncovered areas of floor 1 hr. after cleaning, and the mean counts of settle plates exposed during the same period. The results suggest that airborne contamination accounts for less than half the number of bacteria that were deposited on this floor. The other sources of contamination were probably shoes and trolley wheels; dust blown from adjacent uncleaned areas of floor is another possible source.

Table 1. *Mean bacterial counts from covered and uncovered areas of floor and on settle plates exposed for* 1 *hr.*

	Mean total bacterial counts		
	Impression plates		
Total observations	Covered area	Uncovered area	Settle plates
20	12	*164 ± 21	*63 ± 2·8

* t (18 degrees of freedom) = 4·45. $P < 0·001$.

Accumulation of bacteria on surfaces in 7–9 days

Methods

Two studies were made in a female geriatric ward containing 13 beds, and one study was made in the open section of a female surgical ward containing 14 beds. The surfaces of two vinyl squares (4 sq. ft. in area) were cleaned by mopping with 70 % ethyl alcohol and exposed in the ward; one square was raised 6 in. above the floor, and the other was placed on the floor. The two squares were not cleaned again during the experiment, and the square on the floor was otherwise treated as part of the ward floor. The ward itself was mopped daily with soap and water. Samples were taken from the two surfaces and from the ward floor with two impression plates at 0, 1, 2, 4 and 6 hr., daily for 4 days, and on the seventh day. Some samples were also taken on the 5th and 9th day. After the first day, samples were usually taken 6 hr. after cleaning the ward, but in one of the experiments, in the geriatric ward, samples were taken 1–2 hr. after cleaning.

Results

The total bacterial and presumptive staphylococcal counts from the two vinyl surfaces and from the floor in the geriatric ward are shown in Fig. 1. Total counts show a gradual rise to a peak in about 24 hr.; the vinyl square raised above the floor shows less contamination than the square on the floor during the first 6 hr. After the first 24 hr, no further progressive rise in bacterial counts occurred on impression plates from either of the squares or from the floor itself until after the fourth day. A considerable rise in counts from the two vinyl surfaces but not from the clean floor was found on the seventh day. There was no obvious reason for this increase; the organisms were mainly aerobic spore-bearing bacilli, whereas in the previous samples micrococci had predominated. Staphylococcal counts did not rise after the first 24 hr.

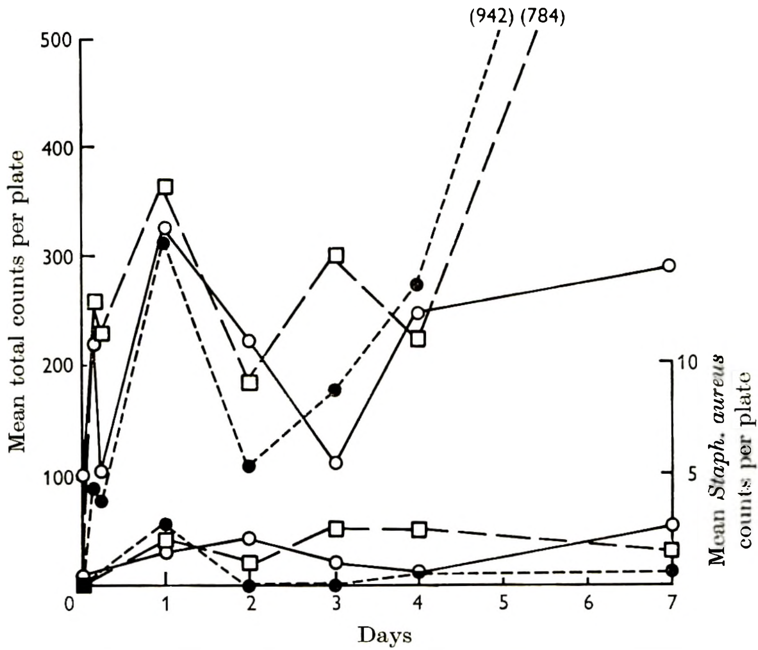

Fig. 1. Mean total and mean *Staph. aureus* counts on impression plates taken at intervals after cleaning a floor in a female geriatric ward during the course of one week. ○——○, Floor; ● - - - ●, vinyl off floor; □ - - - □, vinyl on floor.

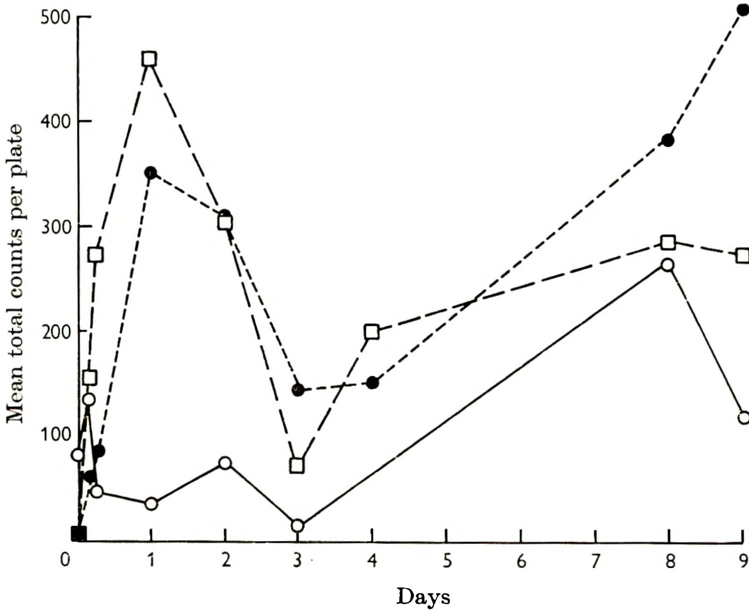

Fig. 2. Mean total counts on impression plates taken at intervals after cleaning the floor in a female geriatric ward during the course of nine days. ○——○, Floor; ● - - - ●, vinyl off floor; □ – – □, vinyl on floor.

The results of a similar experiment are shown in Fig. 2. Counts from the vinyl squares again rose to a peak in 24 hr.; after the peak there was a fall in counts, followed by an increase on all surfaces after the third day. In this experiment samples were taken between 1 and 2 hr. after cleaning the floor; this shorter interval between cleaning and sampling was associated with lower counts on the floor than on the vinyl squares.

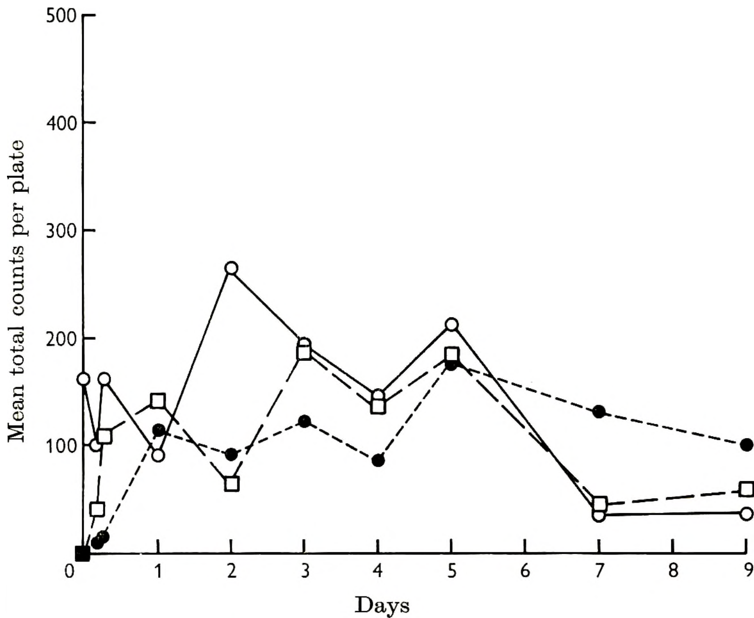

Fig. 3. Mean total counts on impression plates taken at intervals after cleaning the floor in a female surgical ward during the course of nine days. ○——○, Floor; ● - - - ●, vinyl off floor; □ – – □, vinyl on floor.

Figure 3 shows the result of a similar experiment in a female surgical ward. Counts from the two vinyl squares again rose to a peak after 24 hr., and on all three areas they remained at about the same level for 9 days. The count from the floor showed an increase on the second day, due to Gram-negative bacilli surviving on a damp area. Apart from this, most of the counts were less than 200 per plate, which was lower than those usually obtained in the geriatric ward.

The three experiments showed that the bacterial flora of ward surfaces gradually increased over a period of 24 hr. In one experiment the counts remained at approximately the same level for at least 9 days. In the two other experiments, this 'plateau' was rather less obvious because of some unexplained irregularities, but the results over the first 4 days suggest a similar phenomenon, and the counts of *Staph. aureus* in one experiment show the establishment of an equilibrium. The female surgical ward was less crowded than the geriatric ward, and the differences in the equilibrium levels probably correspond with differences in contamination.

Accumulation of airborne bacteria over a period of 5 weeks

Methods

A vinyl square was exposed for 34 days in the female ward, and a similar square was exposed in a male surgical ward during the same period. The squares were raised 3 in. above the floor and were therefore contaminated only with airborne bacteria; they were cleaned with 70 % ethyl alcohol before exposure, and not again during the experiment.

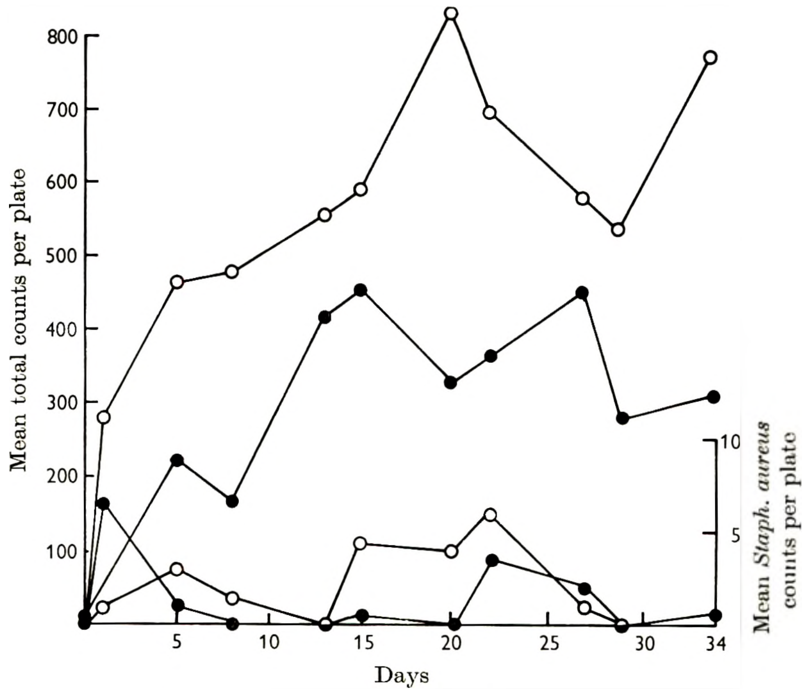

Fig. 4. Mean total and mean *Staph. aureus* counts on impression plates taken from exposed vinyl squares in male and female surgical wards during the course of 5 weeks. ○——○, Male surgical ward; ●——●, female surgical ward.

Results

Figure 4 shows the results. The counts increased gradually over a period of 2–3 weeks, and then remained, with some fluctuation, at approximately the same level. A high proportion of aerobic spore-bearing bacilli was isolated after the first week. It seems that an equilibrium between death (or removal) and deposition of vegetative organisms occurs within 24 hr., and a second equilibrium occurs at a later stage with a predominance of spore-bearing bacilli. Counts of *Staph. aureus* also showed the establishment of a fluctuating equilibrium, but without the further rise shown by counts of total organisms. The difference in bacterial counts in the two wards was associated with differences in the numbers of patients and in activity.

The effect of disinfection of a ward floor on bacterial counts over a three-week period

In a previous study (Ayliffe *et al.* 1966) cleaning with an effective disinfectant (Sudol 1/100) killed or removed 99 % of bacteria from a surgical ward floor, while soap and water killed or removed only about 80 % of the bacteria. Although the effects of both methods of cleaning were apparently annulled by rapid recontamination, it might be expected that cleaning a floor daily with a disinfectant would result in a lower equilibrium level of contamination that that which occurred when soap and water was used for cleaning. A comparison was therefore made of bacterial counts on an area of floor mopped with Sudol 1/100 and an area mopped with soap and water.

Table 2. *Floor bacteria 24 hr. after cleaning with Sudol or with soap and water over a period of 21 days*

Method of cleaning	No. of samples	No. of plates	Mean bacterial count per plate
Soap and water	9	18	824
Sudol 1/100	9	18	848

Methods

Two squares of vinyl were placed on the floor of a female surgical ward in an area where traffic was heaviest. Samples were taken after 24 hr. exposure, two impression plates being taken from each square. For a period of 3 weeks one square was mopped daily with Sudol (1/100) while the other square was mopped at the same time with soap and water. Nine samples were taken from each square before cleaning during the period.

Results

Table 2 shows that there was no appreciable difference in mean counts from the square mopped with Sudol and the square mopped with soap and water.

Death-rate of *Staph. aureus* on surfaces

In a previous section, the establishment of an equilibrium between the death or removal of bacteria and the deposition of bacteria on the surface is described. There are great differences in the death rate of different types of bacteria; Gram-negative bacilli die more readily than Gram-positive cocci when their suspending medium dries, which explains the preponderance of the latter in dry environments (Lowbury & Fox, 1953; F. Pettit, personal communication). Staphylococci can survive for long periods in dust (Lidwell & Lowbury, 1950), but disappear much more rapidly from exposed surfaces in a ward (Skaliy & Sciple, 1964).

In the experiment described here the survival of staphylococci which settled on a vinyl surface from a disperser of the organism was assessed.

Methods

A vinyl square was placed on the floor and cleaned with 70 % alcohol. The disperser exercised for 2 min. near the square, which was then sampled with two impression plates 1 hr. after contamination, daily for 4 days and on the seventh day. The vinyl square was exposed to daylight but not to direct sunlight during the experiment. Four other similar experiments were made, but in two of these experiments a disk of vinyl was contaminated by the disperser and was sampled by the agar cylinder method of Ten Cate (1965) (using 'Agaroid Oxoid') at similar times after contamination.

Results

Table 3 shows a progressive reduction in numbers of *Staph. aureus* isolated from vinyl surfaces in all experiments over a period of 7 days. Although there was some variation between experiments, an appreciable reduction occurred after 24 hr. in four experiments, and few staphylococci were isolated after 4–7 days.

Table 3. *Survival of* Staph. aureus *on a vinyl surface contaminated by a staphylococcal disperser*

Time of sampling after contamination	Total counts of *Staph. aureus* per sample (impression plate) in experiments					Mean % survivors
	1	2	3	4	5	
1 hr.	15	43	29	28	15	100
1 day	6	19	17	2	16	46
2 days	—	17	5	0	1	22
3 days	10	—	3	0	0	12·5
4 days	—	6	0	0	1	6·7
7 days	0	3	0	0	0	1·9

CONTAMINATION OF FLOORS BY SHOES: THE EFFECT OF TACKY AND DISINFECTANT MATS

It has been shown that recontamination after cleaning a floor may occur from settlement of airborne bacteria or from other sources. Barber & Dutton (personal communication) have shown that *Staph. aureus* can be transferred from one area to another on the soles of shoes; they found that organisms were removed from shoes by walking on a mat with a slightly sticky surface ('Takimat'). On the other hand, it has also been found (E. J. L. L., unpublished results) that organisms could be transferred from a contaminated Takimat to clean trolley wheels. In the following experiment, the effect of Takimat and disinfectant mats on the transfer of bacteria by shoes was assessed.

The removal of organisms from a shoe by a Takimat

Methods

Two squares of vinyl, cleaned with 70 % alcohol, were placed one on each side of a Takimat. A subject wearing smooth, rubber-soled shoes walked on an area of

floor contaminated by the disperser, and then stepped on one of the clean surfaces. One shoe was then pressed on the clean Takimat and the other was pressed on the clean area of vinyl. A third step with each shoe was then taken on a clean area of vinyl. The six areas were then sampled with impression plates. The experiment was repeated twice.

Results

Table 4 shows that many organisms were deposited by shoes on the Takimat, but also on the control area of clean vinyl. Larger numbers of *Staph. aureus* were deposited on the mat than on the vinyl surface, but many bacteria were deposited by the third step after treading on the mat; in Expt. 3, more *Staph. aureus* was transferred to the clean floor after stepping on the mat than after stepping on the control area of vinyl.

These experiments also confirm that bacteria, including *Staph. aureus*, may be transferred to floors by contaminated shoes.

Table 4. *Removal of bacteria from a shoe by a Takimat*

(Three experiments)

Floor areas sampled by impression plate	Total bacterial count per plate			*Staph. aureus* per plate		
	1	2	3	1	2	3
Site of first step (on vinyl before stepping on Takimat)	185	76	320	10	3	28
Site of second step (Takimat)	394	145	196	18	7	25
Site of third step (on vinyl after stepping on Takimat)	199	10	194	6	0	26
Controls						
Site of first step on vinyl	358	75	79	9	7	30
Site of second step on vinyl	283	35	82	9	0	3
Site of third step on vinyl	95	20	80	1	0	10
Clean Takimat	16	8	15	0	0	0

Table 5. *The effect of a Takimat at entrance on bacterial counts in room*

	No. of plates	Mean bacterial count per impression plate: In air lock	In cubicle
Takimat in air lock	17	120	*68 ± 14·6
No Takimat in air lock	13	125	*105 ± 27·0

* t (28 degrees of freedom) = 1·29. $P > 0·1$.

The effect of a Takimat on bacterial counts in a side ward

A Takimat was placed in an airlock at the entrance to a plenum-ventilated, single-bedded cubicle, so that anyone entering or leaving the cubicle walked on the mat. The floor of the cubicle and airlock were disinfected daily, 4 hr. before sampling. Two floor impression plates were taken in the airlock at positions 2 ft. proximal to the mat, and two plates were taken in the cubicle at positions 2 ft.

distal to the mat. Samples were also taken in similar positions in another plenum-ventilated cubicle without a Takimat. Four series of samples were taken during one period of 7 days and five series of samples were taken during another period of 7 days after putting down a fresh Takimat. Table 5 shows a slightly (but not significantly) lower mean bacterial count from the floor of a cubicle with a Takimat at the entrance than from the control cubicle without a Takimat.

Transfer of organisms from a contaminated Takimat to a clean floor

A Takimat which had been walked on in the airlock of a ventilated cubicle for 7 days was used in this experiment. The sole (smooth rubber) of a shoe was cleaned with 70 % ethyl alcohol and allowed to dry without touching the floor. The subject wearing the shoe stepped on a clean area of vinyl and then on the contaminated Takimat. Four further steps were then taken on another clean area of vinyl flooring. Samples were taken with impression plates from the floor after a step with the clean shoe; the areas trodden on the first and fourth steps after contamination of the sole of the shoe by the Takimat and the mat itself were also sampled.

Table 6 shows that, although the Takimat was heavily contaminated only a small number of bacteria were transferred on a clean shoe to a clean floor by treading on the dirty mat.

Table 6. *The transfer of bacteria from a contaminated Takimat to a clean floor*

Area sampled by impression plate	Total organisms per impression plate
Floor after contact with clean shoe	2
Contaminated Takimat	1000
Floor after contact with shoe:	
(1) First step after Takimat	31
(2) Fourth step after Takimat	12

Protection of a clean floor by a disinfectant mat in the doorway

A honeycomb type of mat ('Recticel') containing numerous small cells which were filled with a phenolic disinfectant (Hycolin 2 %) was used in the experiment. A subject wearing smooth, rubber-soled shoes walked over an area of floor contaminated by the disperser, and then stepped on an area of floor previously cleaned with 70 % alcohol. He then stepped on the disinfectant mat, and finally took 10 steps on a clean vinyl floor. Impression plates were taken from areas of clean floor on which the subject stepped before he walked on the mat, and also from the areas trodden on his first and tenth steps after walking on the mat. Since the shoes were wet immediately after stepping on the mat and left wet patches on the floor, samples were taken 1 hr. later when the floor was completely dry.

Table 7 shows that the transfer of organisms from a shoe to the floor was reduced on the first step after walking on the mat. An increase was obtained on the 10th step which showed that not all of the bacteria remaining on the shoe were killed. The floor was quite wet under the first tread after stepping on the mat, but much

less so after the tenth tread. The time required for the floor to dry was correspondingly longer after the first tread, which would allow more time to kill the organisms transferred from shoe to floor.

Table 7. *The effect of treading on a disinfectant mat on the contamination of a clean floor by shoes*

Floor area sampled by impression plate	Total bacterial counts and counts of *Staph. aureus* per impression plate from floor			
	Left foot		Right foot	
	Total	*Staph. aureus*	Total	*Staph. aureus*
Before stepping on mat	127	62	194	70
First step after mat	3	0	3	0
10th step after mat	40	7	46	16

The contamination of floors during cleaning

The effect of using contaminated mop-water

In an investigation on the cleaning of ward floors, the use of soap and water was not much less effective in reducing the numbers of bacteria than a disinfectant solution (Ayliffe *et al.* 1966). These results were obtained with a clean mop and water; Walter & Kundsin (1960) have shown that floors may become contaminated if the mop and water are dirty. Gram-negative bacilli are often isolated in large numbers from wet floors but rarely from dry floors.

The effect of using contaminated mop water on bacterial counts from the floor was assessed in the following experiment.

Methods

A male geriatric ward (24 ft. × 48 ft.) containing 13 beds was mopped with soap and water. The mop and bucket were thoroughly rinsed in hot water before use. The ward floor was sampled with six impression plates before cleaning. Three areas (15 in. × 9 in.) of floor were chosen for sampling after cleaning. The first area chosen was cleaned immediately with the clean mop and water, the second after cleaning one-third of the ward, and the third after cleaning two-thirds of the ward. Each area was covered immediately after cleaning with an inverted cardboard box to prevent recontamination by airborne and other bacteria, and sampled with six impression plates after 1 hr. The mop water became increasingly dirty during the cleaning of the ward; total bacterial counts were made from the water before cleaning, and again after cleaning one-third, two-thirds and the complete ward. Five experiments were made with soap and water and one with Sudol (1/100).

Results

Table 8 shows that the mean bacterial count from the floor, cleaned with soap and water, was higher on areas cleaned last than on areas cleaned first. On the floors cleaned with disinfectant, counts were low on all areas. The floor contamina-

tion occurred from the mop or mop water, since the areas were protected from recontamination with bacteria from the air and from shoes.

Table 9 shows an increasing bacterial count in water from the mop bucket during the course of cleaning with soap and water; no comparable increase in total counts occurred when a disinfectant was used.

Table 8. *Bacterial floor counts after mopping ward floor*

	Mean total impression plate counts on covered floor 1 hr. after cleaning with:			
	Soap and water		Sudol 1/100	
Time of sampling floor	No. of plates	Mean total count	No. of plates	Mean total count
Before cleaning	30	337	9	325
After cleaning first area	18	6	6	4
After cleaning one-third of ward	24	32	6	5
After cleaning two-thirds of ward	30	104	6	4

Table 9. *Bacterial counts from mop water during and after mopping a ward floor*

	Mean viable counts on treatment of floor with:			
	Soap and water		Sudol 1/100	
	No. of samples	Mean total counts per ml.	No. of samples	Mean total counts per ml.
Before cleaning	5	10	1	20
After cleaning one-third of ward	5	650	1	10
After cleaning two-thirds of ward	5	15,000	1	30
After cleaning complete ward	4	34,000	1	20

The transfer of bacteria to floor by a contaminated mop

Methods

An area of vinyl floor was 'cleaned' with clean water and a sponge mop that was known to be contaminated with *Pseudomonas aeruginosa*. Impression plates were taken immediately before and after the floor was mopped and at $\frac{1}{2}$, 1, 2 and 3 hr. after mopping. The experiment was repeated on two further occasions. A similar experiment was made after rinsing the mop in a phenolic disinfectant (Hycolin 2 %) and after immersing the mop for 10 min. in the disinfectant.

Results

A heavy confluent growth of *Pseudomonas aeruginosa* and other Gram-negative bacilli was found on plates taken immediately after mopping with a contaminated mop. The counts were considerably reduced in all three experiments during the drying of the floor, and a few colonies were isolated after 3 hr. Rinsing the mop five times in the disinfectant reduced the number of Gram-negative bacilli isolated from the floor, but immersion for 10 min. was required to kill all the organisms.

Redispersal of bacteria from floors into the air

Since it is difficult, and often impracticable, to prevent rapid recontamination of the floors of busy wards, the role of bacteria on floors as a potential source of cross-infection requires consideration. Bacteria-carrying particles on the floor may be redispersed into the air by natural draughts, traffic, or procedures such as bed-making, drawing curtains, sweeping or the use of vacuum cleaners with no filters or with inadequate filters. In the present investigation attempts were made to measure the numbers of bacteria redispersed from a contaminated floor by three methods of disturbance: (1) controlled air movements (blowing with a jet of air from an electric hair dryer), (2) sweeping with a broom, and (3) vigorous movements of a subject who did not disperse *Staph. aureus*.

Methods

An area of vinyl floor (4 ft.^{2}) in a small room was cleaned with 70 % ethyl alcohol, and a preliminary sample of air (50 ft.^{3}) was taken with a slit-sampler. The floor was then contaminated, either by shaking for 2 min. a blanket brought from the bed of a patient with burns colonized by tetracycline-resistant *Staph. aureus*, or by exercise of the disperser near the cleaned area. A second air sample was taken during the period of contamination, and four settle plates were exposed (period 0–30 min.): the settle plates were then replaced by fresh settle plates which were also exposed for 30 min. (period 30–60 min.). One hour after contamination an air sample was taken, and six impression plates were taken from the floor.

Redispersal of bacteria from the floor was then attempted by directing a jet of cold air from an electric hair dryer at the area of maximum contamination for 2 min. ('blowing'); the velocity of the air stream was measured with an anenometer and found to be approximately 245 ft./min., which was greater than any natural air movements found in the wards or the laboratory. Air samples were taken for 1 min. during and 1 min. after blowing. Four settle plates were again exposed (period 60–90 min.) and six impression plates were taken from the floor immediately after blowing. Settle plates were again changed after 30 min. (period 90–120 min.) and a final air sample was taken 1 hr. after blowing. All air samples were of 50 ft.^{3}. To avoid contamination of air by *Staph. aureus* from the clothing of the operator, samples were taken by different members of the laboratory staff during the period of contamination of the floor and during the period of redispersal. None of these operators was a disperser of *Staph. aureus*.

Similar experiments were carried out on terrazzo flooring, and other methods of redispersal were studied—sweeping with a dry broom, and exercise (jumping for 2 min.) by a non-disperser.

Results

Table 10 shows the result of slit-sampling in six experiments. No marked increase in airborne organisms (either total or *Staph. aureus*) occurred during blowing on a vinyl surface (Expts. 1 and 2). An increase in airborne organisms occurred after blowing on a terrazzo floor (Expt. 3), but there was more contamination of air by

Table 10. *Studies on the redispersal of bacteria from the floor into the air*

Time of sampling	Total counts and counts of *Staph. aureus* in 50 ft.^{3} of air (slit-sampler)											
	Expt. 1 (vinyl, blowing)		Expt. 2 (vinyl, blowing)		Expt. 3 (terrazzo, blowing)		Expt. 4 (vinyl, sweeping)		Expt. 5 (vinyl, exercise)		Expt. 6 (terrazzo, exercise)	
	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*
Preliminary sample	358	0	370	0	137	0	720	0	101	0	235	0
During contamination*	†+ + +	8160	7900	2000	8720	2000	5800	2000	2400	460	1136	288
1 hr. after contamination	652	15	364	1	286	8	432	20	92	7	48	0
During floor disturbance	704	24	332	1	458	27	3004	158	169	7	64	1
1 hr. after floor disturbance	464	0	180	0	97	2	32	0	56	0	2	0

* Contamination by shaken blanket in Expts. 2–6; exercise by staphylococcal disperser in Expt. 1.
† Too numerous to count.

Table 11. *Studies on the redispersal of bacteria from the floor into the air*

Total counts and counts of *Staph. aureus* on four settle plates exposed for 30 min.

Time of sampling	Expt. 1 (vinyl, blowing)		Expt. 2 (vinyl, blowing)		Expt. 3 (terrazzo, blowing)		Expt. 4 (vinyl, sweeping)		Expt. 5 (vinyl, exercise)		Expt. 6 (terrazzo, exercise)	
	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*
During and after contamination* (0–30 min.)	2136	1004	638	89	832	65	544	99	129	43	68	12
After contamination (30–60 min.)	20	0	18	0	7	1	30	1	5	1	5	0
During and after floor disturbance (60–90 min.)	41	1	39	2	56	4	222	32	2	0	10	0
After floor disturbance (90–120 min.)	19	1	31	1	6	0	16	0	3	0	3	0

* Expt. 1. Contamination by staphylococcal disperser. Expts. 2–6. Contamination by shaken blanket.

Table 12. *Studies on the redispersal of bacteria from the floors into the air**

Mean total counts and counts of *Staph. aureus* per floor impression plate

Time of sampling	Expt. 1 (vinyl, blowing)		Expt. 2 (vinyl, blowing)		Expt. 3 (terrazzo, blowing)		Expt. 4 (vinyl, sweeping)		Expt. 5 (vinyl, exercise)		Expt. 6 (terrazzo, exercise)	
	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*	Total	*Staph. aureus*
Before floor disturbance	+ + +	1240	105	26	390†	14	97‡	21	104	16	75	1
After floor disturbance	+ + +	1093	112	34	234†	8	31‡	7	116	15	95	1

* Expt. 1. Contamination by staphylococcal disperser. Expts. 2–6. Contamination by shaken blanket.
† t (10 degrees of freedom) = 2·79, $P < 0{\cdot}02$.
‡ t (10 degrees of freedom) = 5·26, $P < 0{\cdot}001$.

sweeping a vinyl floor (Expt. 4). A small increase in total organisms (probably from the subject exercising), but not *Staph. aureus*, occurred during the exercise of a non-disperser (Expt. 5). A similar result was obtained by exercising on a terrazzo floor. The results of the corresponding settle-plate counts are shown in Table 11 and confirm the slit sampling results. Table 12 shows the mean impression plate counts taken before and after attempts at redispersal. Reductions in total organisms and in *Staph. aureus* on the floor were obtained in Expts. 3 and 4; the reduction corresponded to the increase in counts of airborne bacteria. These results show that, in spite of the heavy initial airborne contamination, few staphylococci were redispersed into the air by blowing on the floor or by a subject exercising, More organisms were raised by blowing on a terrazzo than on a vinyl floor, but this redispersal was much smaller than that obtained by sweeping with a broom.

CONTAMINATION OF WALLS

Wypkema & Alder (1962) and Froud, Alder & Gillespie (1966) found little contamination of walls and even less contamination of ceilings in hospital wards and operating theatres. These findings were supported in general by results of the study summarized below, but we describe certain conditions where walls may become heavily contaminated.

Accumulation of bacteria on the wall of an operating theatre

Methods

After a theatre wall had been thoroughly washed with soap and water, an area of 13·5 ft.^{2} was marked off and left uncleaned for 12 weeks. The remaining area of wall was cleaned weekly with a fresh oiled ('Kex') mop (Babb *et al.* 1963). Ten impression plates were taken each week from the uncleaned wall and from the adjacent area of clean wall.

Table 13. *Bacterial contamination of walls in an operating theatre*

Time of sampling (after washing)	Mean counts from 10 impression plates on area: Left intact after washing		Cleaned weekly with oiled mop	
	Total	*Staph. aureus*	Total	*Staph. aureus*
1 day	2·8	0	3	0
1 week	5·0	0	6·4	0
2 weeks	3·4	0·6	2·8	0
3 weeks	3·4	0·2	7	0·2
4 weeks	3·2	0	1·8	0·2
5 weeks	4·6	0·2	1·6	0
12 weeks	1·2	0	1·4	0
1 day after second wash	1·0	0	0·8	0

Results

Table 13 shows the mean count of organisms and of presumptive *Staph. aureus* on impression plates from the two areas of wall. Counts were low, and there was no

evidence of an increase in contamination during the period of study or of any significant difference between the cleaned and uncleaned areas of wall. The results suggest that a 'plateau' is obtained on wall surfaces, but with a much lower level of contamination than that found on floors.

Bacteria in areas of bare plaster

Impression plates were taken from areas of clean paintwork under the window of an operating theatre. The walls were moist with condensate and included patches of wet exposed plaster. Plate 1 shows the appearance of this wall and of an impression plate taken from it. After overnight incubation, large numbers of bacteria, including many Gram-negative bacilli, had appeared on the impression plate in areas which corresponded with the position of the bare plaster. Similar samples taken from dry plaster exposed on an inner wall yielded very small numbers of bacteria.

Staphylococcal contamination of a wall by fingers of a disperser and the transfer of organisms from these areas by the fingers of a non-carrier

Walls are often touched by contaminated fingers, and it is possible that pathogens may be picked up from such areas by the hands of others and transferred directly or indirectly to patients. An assessment of this hazard was made in the following experiment.

Table 14. *Transfer of* Staph. aureus *from contaminated wall by fingers of non-carrier*

Area of wall sampled	Fingers	Total *Staph. aureus* per plate
Contaminated by disperser	Left 1	21
	Right 2	32
Contaminated by transfer on fingers of non-carrier	Left 3	5
	Right 3	2

Methods

The fingers of both hands of the staphylococcal disperser were sampled directly by impression on an agar plate. Two sets of imprints (L. 1 and L. 2, R. 1 and R. 2) from the four fingers of each hand were made by firm pressure on a tiled wall previously cleaned with 70 % alcohol. The disperser's fingers were again sampled on an agar plate. Two of the four contaminated areas were then sampled with an impression plate (L. 1 and R. 2). The fingers of a non-carrier of staphylococci were now sampled on an agar plate, after which the remaining two contaminated areas of the wall were firmly touched by the fingers of both hands of the non-carrier (L. 2 and R. 1). A clean area of the wall was then firmly touched by the contaminated fingers of the non-carrier. These two areas of wall were sampled by impression plates L. 3 and R. 3. Finally the non-carrier's fingers were sampled by impression on an agar plate.

Results

Table 14 demonstrates the contamination of the wall by the fingers of the disperser (L. 1 and R. 2). *Staph. aureus* was also transferred from the contaminated wall to another area of wall by the fingers of the non-carrier (L. 3 and R. 3), but in much smaller numbers; these staphylococci showed the same antibiotic sensitivity pattern as the organisms isolated from the disperser. Table 15 shows that smaller numbers of *Staph. aureus* were deposited on an agar plate by the disperser after he had touched the wall than before. No staphylococci were isolated from the fingers of the non-carrier either before contaminating the fingers or after transferring staphylococci to a clean area of the wall.

Table 15. *Staphylococcal contamination of fingers of the disperser before and after touching a wall*

Time of sampling	Fingers of disperser	Total *Staph. aureus* per plate
Before contaminating the wall	Left	*ca.* 100
	Right	*ca.* 100
After contaminating the wall	Left	87
	Right	66

Table 16. *Survival of* Staph. aureus *on a wall contaminated by fingers of the disperser*

Time of sampling (after contamination)	Total bacterial counts and counts of *Staph. aureus* per impression plate			
	Expt. 1 (dry fingers)		Expt. 2 (wet fingers)	
	Total	*Staph. aureus*	Total	*Staph. aureus*
1 hr.	12	9	1040	560
1 day	4	0	151	55
2 days	0	0	6	0
3 days	2	0	4	0
4 days	0	0	3	0
7 days	0	0	0	0

Survival of Staph. aureus *on walls*

In view of the heavy local contamination of walls that may occur where they are touched by the hands of the staphyloccocal carrier, experiments were made to assess the survival of *Staph. aureus* on a wall contaminated by the fingers of the disperser.

Methods

Six areas of a glossy tiled wall were contaminated by the fingers of the disperser. Areas 1 to 6 were contaminated in sequence with one hand and the same areas in reverse order 6 to 1 with the other hand so that all areas were contaminated by the fingers of both hands. Dry fingers were used for contaminating the wall in the

first experiment and wet fingers in the second experiment. The areas were sampled in a random order with impression plates 1 hr. after contamination, and then daily for four days and on the seventh day.

Results

Table 16 shows that organisms from fingers, especially *Staph. aureus*, die rapidly on a clean surface. No colonies of *Staph. aureus* were isolated after 2 days. The other organisms isolated were mainly *Staph. albus*. Expt. 2 showed that considerably greater contamination of the wall was obtained with wet fingers.

DISCUSSION

Uncontaminated floors of hospital wards rapidly acquire bacteria from the environment, but after short periods, which vary with the amount of recontamination, the removal and death of bacteria approximately balance the addition of bacteria from the environment. An earlier study (Ayliffe *et al.* 1966) showed that cleaning the floor with a disinfectant (Sudol 1/100) caused a significantly greater reduction in bacterial flora than washing with soap and water when the area was protected from recontamination. Although the effect of cleaning was largely annulled by recontamination, it seemed likely that more effective cleaning would lead to a lower equilibrium level of bacteria on the floor; such a difference was not, however, found in a comparison of contamination levels 24 hr. after cleaning on an area of ward floor cleaned daily with soap and water and a similar area cleaned daily with Sudol (1/100).

From this study we deduce that at most times daily disinfection contributes little or nothing to the bacteriological cleanliness of ward floors. In operating theatres and other areas with less contamination than that which occurs in wards, disinfection or cleaning might be expected to be more effective. The main function of disinfection, however, must be in the removal of sporadic local contamination which occurs when floors or walls become contaminated with sputum, pus, urine and other fluids, or when walls are touched by fingers of a heavy carrier of pathogens. Since the occasions when such contamination occur often pass unnoticed, there is a case for regular disinfection to prevent this sporadic hazard in areas where the risk of contamination is high. Disinfectants also help to prevent a build-up of bacterial contamination in a bucket of water used for cleaning a floor. Gram-negative bacilli are the predominant flora of mop buckets and mops which have not been disinfected after use; though most of the organisms die during the evaporation of the water from the floor, the surface remains wet and heavily contaminated for some time after washing. Neither washing nor disinfection can be expected to remove the heavy bacterial colonization that is found on moist areas of exposed plaster of walls, or on damaged floor surfaces; to remove this hazard the surfaces must be repaired and a new finish applied. In view of the small contamination usually found on walls and the damage that may be caused by frequent washing, there is in fact a case for reducing the frequency of washing walls.

Both contact and airborne contamination of floors were demonstrated in these studies. Since disinfection and other methods of cleaning have limited value in

reducing the numbers of bacteria on floors, it is clearly desirable to prevent contamination of these surfaces. Tacky and disinfectant mats appeared to have very limited value in preventing the transfer of bacteria on shoes; they are clearly no substitute for over-shoes or rubber boots reserved for use in the clean areas. Tacky mats become dirty in a short time, and slippery patches are found on the floor adjacent to disinfectant mats. Transfer of bacteria on hands of carriers to walls and other surfaces can be reduced by washing the hands with antiseptic detergent preparations, and by the use of rubber or plastic gloves in handling infected patients; these measures may, however, fail in the case of heavy dispersers of *Staph. aureus*, who are also likely to contaminate the air with large numbers of staphylococci. In the absence of routine surveillance, a source will not be recognized unless it causes an outbreak of infection; where 'high-risk' patients are under treatment it is clearly desirable to forestall this hazard by surveillance, and also to use various methods to prevent dispersal of staphylococci, including special clothing (Bernard *et al.* 1965), and bathing with hexachlorophane detergent preparations.

The importance of bacteria on floors and walls as a source of infection is not clearly defined. *Staph. aureus* deposited either by settlement from air or by contact disappeared in a few days from contaminated surfaces, but in that time it may be a source of cross-infection. Experiments with radioactive dust (Brunskill, 1966; Jones & Pond, 1966) and with bacterial markers (Carson, 1966) have shown small amounts of redispersal of settled dust. Our failure to redisperse settled bacteria into the air from a vinyl surface and the small numbers redispersed from a terrazzo surface are in keeping with previously reported failures to reduce infection by the oiling of floors (Clarke *et al.* 1954). But while these experiments support the view that floor dust is not an important source of airborne infection, contact transfer (e.g. by toys dropped on the floor, or to nurses' hands when putting on overshoes) may be a sporadic source of infection. Walls and doors, which acquire much smaller levels of bacterial contamination than floors, may be heavily contaminated by sporadic contact (e.g. with fingers of a staphylococcal carrier); since such contamination is likely to occur in areas touched or handled by many people, it may be an important cause of infection from the inanimate environment.

SUMMARY

Impression plates from initially clean horizontal surfaces and floor areas in surgical wards showed a rapid accumulation of bacteria, mainly micrococci, which reached a fluctuating equilibrium after about 24 h. A later increase in bacterial contamination (mainly with aerobic sporing bacilli) to a higher equilibrium level after about 14 days occurred on uncleaned areas. Walls, even if left unwashed, acquired very few bacteria, but many were deposited locally when the wall was touched by a subject whose skin carried large numbers of staphylococci; moist exposed plaster was also heavily contaminated.

Regular use of a disinfectant ('Sudol' 1 in 100) in cleaning a ward floor did not reduce the equilibrium level of bacteria on the floor.

The transfer of staphylococci from contaminated to clean areas on the soles of shoes was demonstrated; the use of tacky and disinfectant mats did not appreciably reduce the transfer of bacteria by this route.

Staphylococci deposited on a wall by a disperser were shown to be transferred from the contaminated area of wall to the hands of another subject who did not previously carry the organism; this subject was shown to transfer the staphylococcus to a wall which he touched.

Attempts to redisperse by air movement *Staph. aureus* which had been shed by a disperser or by a contaminated blanket on to the floor surfaces had little effect; neither blowing with a hair dryer nor brisk exercise appeared to lift any of the staphylococci from a vinyl surface, and only small numbers were lifted by these measures from a terrazzo surface.

The hazards of infection from the inanimate environment are discussed.

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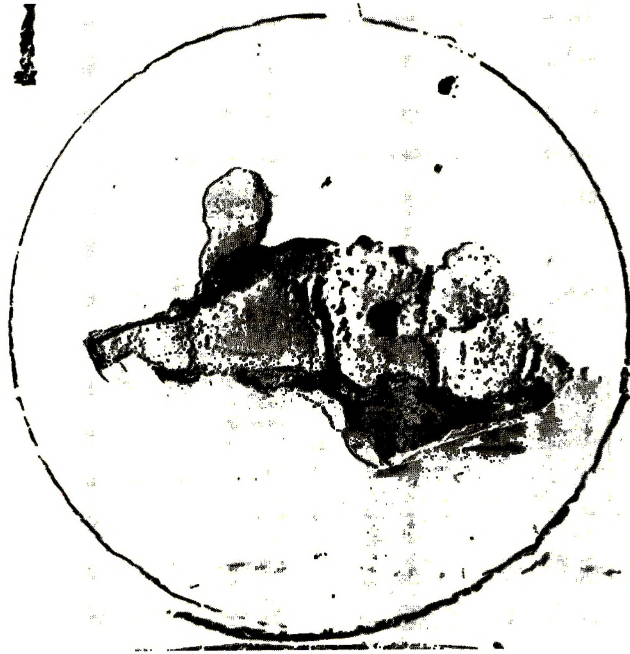
EXPLANATION OF PLATE

A. An area of moist exposed plaster on the wall of an operating theatre from which paint had flaked.

B. An impression plate from the same area showing heavy bacterial growth.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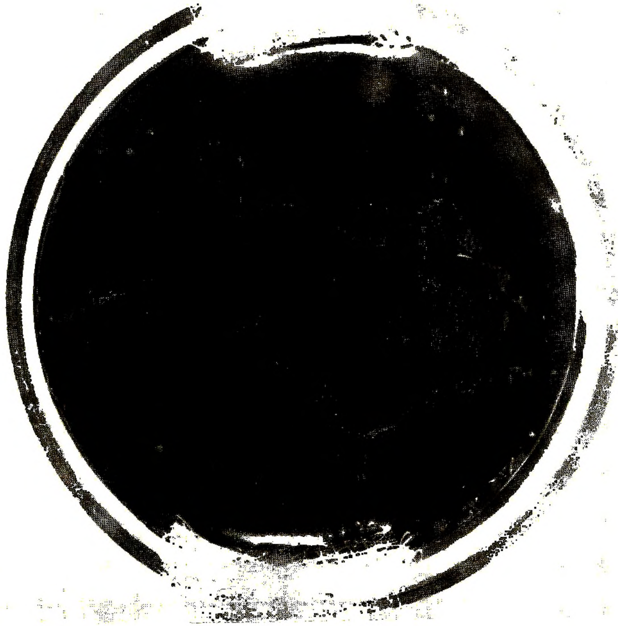
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Studies on the epizootiology of rinderpest in blue wildebeest and other game species of Northern Tanzania and Southern Kenya, 1965–7

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Detailed serological evidence presented by Plowright & McCulloch (1967) showed that extensive infections of rinderpest occurred in various populations of blue wildebeest (*Connochaetes taurinus albojubatus* Thomas, syn. *Gorgon taurinus taurinus* Burchell) from N. Tanzania and S. Kenya over the period 1959–62. However, sera from young wildebeest born and collected in 1963 lacked antibodies attributable to rinderpest infection and it appeared possible that epizootics of the disease had ceased.

Work of this nature lapsed during the whole of 1964, but in January 1965 it became possible to resume the collection of wildebeest material. Attempts were made to re-establish the age distribution of animals with rinderpest antibody in the populations previously examined. Although there had been no report of rinderpest in either cattle or game from the area under consideration during 1964, in view of the occurrence of subclinical rinderpest in wildebeest (Plowright, 1963) it was considered necessary to examine animals of all ages.

In December 1965 and March 1966 field outbreaks of bovine rinderpest were confirmed in N. Tanzania from a district where rapid extension to game species was a distinct possibility. Considerable interest attached to the origin of these outbreaks as they brought to a close a 3-year period (1963–5) during which no rinderpest had been detected in the whole of Tanzania. The scope of the present study was therefore enlarged in an attempt to gather evidence of a prolonged maintenance role by local game.

MATERIALS AND METHODS

Sera

Free-flowing blood was usually obtained from the severed jugular vessels. Serum was separated within 24–72 hr. of shooting, containers in the meantime having been kept as cool as possible. Samples were stored at -20° C., and, where necessary, were transported rapidly on ice.

Neutralization tests

Samples of undiluted sera, previously inactivated at 56° C. for 30 min., were screened for rinderpest-neutralizing antibody in a 5-tube test using $10^{1\cdot 2}$–$10^{2\cdot 8}$

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TCID 50 of culture-attenuated virus per tube (Plowright, 1962). Samples in which neutralizing activity could be detected were subsequently titrated against a virus dose of $10^{1.8}$–$10^{2.6}$ TCID 50 per tube. Details of the methods and controls employed have been described by Plowright & Ferris (1961) and Plowright (1962). Log_{10} SN 50 titres were calculated by the method of Thompson (1947).

Ageing

Watson (1967) described a reliable method for determining the age of wildebeest based on the number of erupted incisor and cheek teeth, or the amount of wear in full-mouthed animals. Alternatively, females may be aged by counting scars left by corpora lutea of pregnancy. Due to regular annual conception by mature females a good correlation has been found between the two methods; scar counting, however, will underestimate the age of an abnormal individual which fails to conceive in any year of adult life. It was only necessary to use this alternative method of ageing for a small proportion of Serengeti migrant wildebeest shot in 1965.

Species other than wildebeest were aged from a general consideration of dentition, body weight and body measurements, bearing in mind the strictly seasonal nature of the reproductive cycle.

Animal populations

With the exception of the Kajiado group, sera were collected from each of the wildebeest populations described by Plowright & McCulloch (1967). Figure 1 of these authors outlines the topography of the area where the current work was undertaken and the wildebeest populations which were sampled.

In connexion with the 1965/6 field outbreaks of rinderpest it was necessary to collect sera from buffalo, eland, impala, warthog, and resident wildebeest within the Loliondo district of N. Tanzania. The distribution of these animals has not been previously mentioned.

Loliondo resident wildebeest, numbering some 5000 animals, have a wet season range immediately to the south of Loliondo township, and in the dry season disperse into the Loita Hills. Additional small groups are to be found during the wet season in the Loita Hills and eastwards towards Lake Natron. Between December and June the resident population can be greatly augmented by Serengeti migratory animals occupying the northern extremity of their wet season range.

The other species mentioned form loose aggregations of herds or family groups throughout the area.

RESULTS

Serengeti migrant wildebeest

A total of 149 sera were examined between January 1965 and April 1966 (Table 1).

Antibodies were found in four samples from 1- to 4-month-old calves born in early 1965 or 1966, but none could be detected in sera of calves born in January 1965 and aged 5–11 months when shot. In three instances it was possible to demonstrate antibodies in the sera of both dam and offspring. It was inferred from

these results that passive transfer of protection took place between cows possessing antibody and their calves, and that this protection was short-lived.

No evidence of active antibody production could be demonstrated in any of 60 samples from animals born since 1963. In contrast rinderpest-neutralizing antibodies were present in 59 of 73 samples (81 %) from animals born before 1962. It seems probable that the last major rinderpest infection of this population occurred in 1961, as approximately 64 % of the animals born in January of that year experienced the disease compared with only 6 % of animals born in 1962. The single positive sample from 1962 was aged by the scar-counting technique.

Table 1. *Distribution of rinderpest antibody in Serengeti migrant wildebeest*, 1965/6

	Year of sampling			
Year of birth	1965	1966	Totals	% positive
In or before 1959	37/45*	2/2	39/47	83·0
1960	12/14	1/1	13/15	86·6
1961	7/9	0/2	7/11	63·6
1962	1/12	0/4	1/16	6·2
1963	0/18	0/2	0/20	0·0
1964	0/5	0/8	0/13	0·0
1965	3/13†	0/12	3/25	0·0‡
1966		1/2†	1/2	
Totals	116	33	149	

* No. positive/no. tested.
† Calves aged 1–4 months old; antibody presumed to be passively acquired.
‡ Percentage derived from 1966 sample.

Table 2. *Distribution of rinderpest antibody in the Kirawira, Mara and Ngorongoro resident wildebeest populations*

		Year of birth							
Population	Collection date	1960	1961	1962	1963	1964	1965	1966	Totals
Kirawira	Mar. 1966	4/9*	0/3	0/4	0/2	0/7	0/5	1/3†	33
Kirawira	Feb. 1967	0/1	1/1	—	0/1	0/1	0/4	0/19	27
Mara	Jan. to Mar. 1966	10/12	3/4	2/13	0/11	0/13	0/7	1/3†	63
Ngorongoro	Apr. 1966	9/10	1/1	4/6	0/1	0/6	0/5	1/1†	30

* No. positive/no. tested.
† Calves aged 2–4 months old; antibody presumed to be passively acquired.

Resident wildebeest populations

The distribution of rinderpest-neutralizing antibody in three non-migratory populations is presented in Table 2. Antibodies found in young calves were in each instance considered to have been passively acquired.

No evidence of recent infection in animals of the Kirawira resident group could be found among samples collected in March 1966 and February 1967; the last outbreak in this population apparently took place in 1961.

Mara residents were sampled in the first 3 months of 1966. Some 81 % of the animals born in early 1960 and 1961 possessed antibody, together with a much smaller proportion (*ca.* 15 %) of animals born in early 1962. There was no indication of infection in any subsequent year.

Antibodies were found in resident wildebeest from the Ngorongoro crater born in 1960, 1961 and 1962, but animals born since January 1963 have remained susceptible. All samples from this population were taken in April 1966.

Field rinderpest

In November 1965 material submitted from cattle in the Loliondo district of N. Tanzania was found to contain rinderpest virus (W. P. Taylor, unpublished). Reports indicated that the disease affected young stock only without causing mortality. Resident wildebeest and other rinderpest-susceptible ungulates (Scott, 1964) were to be found in close proximity to the primary cattle outbreak, and moreover the disease focus was situated on the eastern edge of the wet season range of the Serengeti migrant population. By this time it was known that this highly mobile group of animals, then some 50 miles to the south-east, contained numerous susceptible individuals.

Table 3. *Distribution of rinderpest antibody in various game species from Loliondo District, N. Tanzania, December* 1965 *to January* 1966

Species	Collection date	Year of birth						Totals
		≤ 1960	1961	1962	1963	1964	1965	
Resident wildebeest	Dec. 1965	4/6*	—	0/2	1/2	1/7	0/5	22
Buffalo	Jan. 1966	6/7	—	0/1	1/1	0/1	—	10
Eland	Dec. 1965	0/4	—	1/3	0/2	0/4	0/7	20
Impala	Dec. 1965	0/6	—	0/1	0/2	—	0/7	16
Warthog	Dec. 1965	—	—	—	0/1	—	0/1	2

* No. positive/no. tested.

Sera were obtained from buffalo, eland, impala, warthog, and Loliondo resident wildebeest some 3–6 weeks after the disease was first diagnosed. Results of examination for rinderpest-neutralizing antibody are given in Table 3. It was apparent that during this interval there had been no rapid extension of rinderpest to contiguous game populations. Of the 70 sera examined positive samples were predominantly among buffalo and wildebeest born in or before 1960. These animals were probably infected early in life as there was no evidence of widespread infection between 1962 and 1965 in any of the species from which samples were taken. In this connexion it should be noted that buffalo infected with rinderpest were reported close to Loliondo district in 1959 (Thomas, 1960) and 1960 (Kinloch, 1961). The single positive eland born in 1962 could also have encountered the disease while young, as it has been demonstrated that rinderpest existed that year, albeit in wildebeest, some 70 miles south at Ngorongoro (Plowright & McCulloch, 1967).

Neutralizing antibody was found in the serum from 1 of 2 wildebeest born in 1963 and from 1 of 7 born in 1964 (Table 3). At the time it was impossible to decide whether these animals had been infected by an extension of the cattle outbreak into wildebeest, or whether their antibody had been acquired at a much earlier stage in life. The same problem was posed by the findings of antibody in a 2-year-old buffalo.

In March 1966 a fresh focus of rinderpest was confirmed, still within cattle of the Loliondo district, but in an area where both Serengeti migrant wildebeest and local resident animals were now concentrated. Additional samples were obtained from both populations some three weeks after this second outbreak was detected. As the migrants had moved about 20 miles south there was no difficulty in differentiating the two types of wildebeest. The distribution of antibodies in the 33 migrant wildebeest collected has already been described (see Table 1, results for 1966), while results for the further 21 resident wildebeest are given in Table 4. There were no indications that the bovine field strain of virus had spread to either population: of the 54 samples the only antibodies found were in the sera of three old Serengeti migrant animals, a 3-month-old calf from this group and a 6-year-old resident animal.

Table 4. *Distribution of rinderpest antibody in Loliondo resident wildebeest, April* 1966

Year of birth						
1960	1961	1962	1963	1964	1965	Total
1/1	—	0/2	—	0/9	0/9	21

Table 5. *Titration results for rinderpest neutralizing antibody in sera from Serengeti migrant wildebeest*

Age group* (years)	No. of samples	Mean titre and standard deviation	Range	t Test†
> 12	7	0.86 ± 0.25	0.6–1.4	$t = 1.989$, $p > 0.05$; < 0.1
10–12	6	1.12 ± 0.37	0.8–1.6	$t = 1.248$, $p > 0.2$; < 0.4
7–9	14	0.97 ± 0.59	0.4–2.2	$t = 0.5517$, $p > 0.5$
6	9	1.11 ± 0.38	0.8–1.6	$t = 1.354$, $p > 0.1$; < 0.2
5	8	0.85 ± 0.26	0.6–1.4	$t = 0.1122$, $p > 0.5$
4	7	0.83 ± 0.54	0.4–1.8	—

* All samples collected during 1965.
† Mean titre for each age group compared to that of 4-year-old animals

The antibodies detected in two young wildebeest in the earlier collection of Loliondo resident samples apparently did not represent the commencement of a fresh wildebeest epizootic. It was concluded that these antibodies were acquired before November 1965 and that they were present in only a small proportion of the resident population.

Titration results

Table 5 shows the results of neutralizing-antibody titrations carried out with sera from different age groups of Serengeti migrant wildebeest. When mean titres were compared it was found that antibody levels did not decline with age; however, older animals were undoubtedly exposed to rinderpest virus on more than one occasion.

Animals of the 4-year age group were probably infected in late 1961 (Plowright & McCulloch, 1967). Taking the date of first antibody appearance as January 1962, demonstrable antibodies had persisted for $3\frac{1}{2}$–4 years in the absence of re-infection. Moreover, antibody levels were not significantly lower ($t = 1{\cdot}17$, $P < 0{\cdot}4$, $> 0{\cdot}2$) when compared to figures obtained from the same group of animals some 3 years earlier (Plowright & McCulloch, 1967).

DISCUSSION

The present survey has demonstrated quite clearly that each of the numerically important wildebeest populations of the Serengeti-Mara region, in S. Kenya and N. Tanzania, has been free of rinderpest infection over the last 3 or 4 years. In reviewing the history of rinderpest in wildebeest of this area Plowright & McCulloch (1967) were able to find evidence of continuously recurring infection in the period between 1947 and 1960, while their own results provided evidence for continuing infection up to 1962. However, the recovery of strains of the virus from cattle in N. Tanzania as recently as March 1966 indicates the continuing risk of further disease in these great herds. From the data of Watson (1967) it is possible to calculate that in the Serengeti migrant population there are at present some 200,000 rinderpest-susceptible individuals, amounting to over 60 % of the total. It may be assumed that a similar proportion of each resident population in this area is also at risk. Thus a fresh epizootic of virulent rinderpest in any of these groups could well cause extremely severe losses with the attendant danger of widespread physical dissemination of the virus, and spread to other species.

When the 1965 and 1966 isolations of rinderpest virus were made in the Loliondo area the only other known focus of the disease in East Africa was some 200 miles to the north, in the Isiolo district of Kenya. Strains derived from the Kenya outbreak caused severe clinical signs and some mortality in experimental cattle, whereas the Loliondo strains produced a relatively mild syndrome and no deaths (W. P. Taylor, unpublished). Aside from any difference in strain characteristics it was most unlikely, if not impossible, for infection to have spread between the two localities. Mild rinderpest was reported from the Loliondo district up to the end of 1960 (Roe, 1962) and was still present in 1961 at which time it was reputed to be difficult to detect and to cause only mild symptoms in calves (Branagan & Hammond, 1965). Although a thorough rinderpest vaccination programme was carried out in this district in 1964 (D. Branagan & J. A. Hammond, personal communication) it is suggested that the recent outbreaks were evidence of an enzootic infection that had persisted since 1961 in an undetected form in this isolated part of N. Tanzania.

On this basis an explanation can be given for the findings of antibody in the sera of 2- and 3-year-old wildebeest from the Loliondo resident population. Only two such individuals were detected in 36 samples (Tables 3, 4) taken from animals born between 1962 and 1965, so apparently no widespread infection occurred within the population during this period. However, if small groups of animals had encountered infection, possibly when the population was dispersed during a dry season, it would then be possible to sample aggregations during the wet season and find a small number of animals with neutralizing antibody; this is in fact what was done.

At the time of the second field outbreak large numbers of resident and migrant wildebeest were grazing land from which infected stock had recently been withdrawn. Moreover, sick cattle, although in quarantine, remained localized within the territory occupied by wildebeest (P. Jenkins, personal communication). It was therefore somewhat surprising to find that transmission to wildebeest had not occurred. Until further sera can be obtained from young buffalo the epizootiological role of this species in the Loliondo area will remain uncertain.

In general our survey results confirmed and extended the findings of Plowright & McCulloch (1967). From material collected in 1963 they were able to show that Kirawira resident wildebeest had been infected in 1961 but not in 1962. It is now apparent that no rinderpest infection has occurred in this group of animals in the 5-year period 1962 to 1967.

Plowright & McCulloch (1967) found that wildebeest of the Mara group suffered infection during 1959 but not within the first 8 months of 1960. The present results show that this population was subjected to at least one further rinderpest episode, as animals born in early 1960 and 1961 were found to possess actively acquired antibody. Two of 13 animals aged 4 years at the time of shooting had also experienced the disease. Although both these animals were ascribed to the 1962 calf crop, calving in this population is actually spread over a 2–3 months period between December one year and February of the following year (Watson, 1967). It is suggested that these individuals were born and infected in late 1961 when rinderpest was concurrently infecting yearling animals born earlier the same year. It is of course possible that 1961 yearlings, together with small numbers of very young calves, were infected in the first months of 1962; however, the former interpretation gains support from a report of rinderpest in yearling wildebeest of this population in 1961 (Anon, 1962). Maternally derived immunity would probably have protected the majority of the 1962 calf crop for the first 4–6 months of life (Plowright & McCulloch, 1967), so had the outbreak occurred after the loss of this protection a higher proportion of animals with antibody could reasonably have been expected.

In August 1962 Plowright & McCulloch (1967) found antibodies in the sera of three 9-month-old calves in the Ngorongoro Crater, and in two 8-month-old calves from the same population collected on the Ol Balbal Plains; these antibodies were considered to have been actively acquired. When a further series of samples was collected from the crater in 1963 they were unable to repeat their previous finding yet we were able to show that some 67 % of Ngorongoro wildebeest born in 1962 had experienced the disease (Table 2). The coincidence of our result with the

former result of Plowright & McCulloch (1967) clearly established that this group of animals was last infected in 1962.

There is a remarkable agreement between the present survey and that of Plowright & McCulloch (1967) with respect to the percentage of animals possessing neutralizing antibody in the 1959, 1960, and 1961 calf crops of Serengeti migrant wildebeest. While they were able to detect discrete epizootics in 1960 and 1961 they were uncertain whether or not small numbers of these wildebeest were infected in 1962. In the current work there was no evidence of widespread infection in animals of the 1962 calf crop, and in view of the method used to age the donor of the single positive sample it is entirely possible that no infection occurred during 1962 in the migrant group.

Throughout this report it has been assumed that antibody found in the serum of 1- to 4-month-old calves was passively acquired. This interpretation is in agreement with the work of Plowright & McCulloch (1967) where it was shown that young wildebeest acquired antibodies from their dams within the first few days of life, and that thereafter this antibody decayed by 50 % every 4·4 weeks.

Duration of neutralizing antibody in wildebeest has not been studied hitherto, although Plowright, Laws & Rampton (1964) showed that the hippopotamus (*Hippopotamus amphibius* Linnaeus) retained rinderpest antibody for 18 years following a single infection. The absence of a detectable decline in antibody levels in wildebeest between 1962 and 1965 indicates they may persist for some considerable time to come.

SUMMARY

A serum neutralization test was used to determine the incidence of rinderpest antibodies in populations of blue wildebeest (*Connochaetes taurinus albojubatus* Thomas) occurring in the Serengeti-Mara districts of N. Tanzania and S. Kenya. By correlating the age of animals at the time of shooting with the presence or absence of antibodies it was possible to outline the course of rinderpest in these animals over the period 1961–7.

Serengeti migrant wildebeest were extensively infected in 1961, but not in 1962 or any subsequent year. No samples have been collected from this group since mid-1966. Kirawira resident wildebeest were last infected in 1961 according to samples collected in February 1967. Two of 13 animals born into the Mara resident population in late 1961 or early 1962 were found to possess antibodies, together with a high proportion (74 %) of animals born in early 1961. This finding was considered indicative of a rinderpest epizootic in this group in the terminal weeks of 1961. No subsequent infection occurred in Mara wildebeest between 1962 and March 1966. Ngorongoro resident wildebeest were infected in the second half of 1962 but have remained free of rinderpest from 1963 until at least April 1966.

The findings of the present survey were compared and contrasted with results of a similar study carried out some 2 to 3 years previously.

Field strains of bovine rinderpest virus were isolated from the Loliondo district of N. Tanzania in November 1965 and March 1966. Serum samples from buffalo, eland, impala, warthog, and resident wildebeest which inhabited areas adjoining

the two outbreaks failed to provide evidence of any recent epizootic in game animals. Circumstantial evidence indicated that the virus could have persisted in the Loliondo area since 1961. Two resident wildebeest born in 1963 and 1964 respectively, were thought to have been infected during a period when the disease went unrecognised. Serengeti migrant wildebeest were not found to have been infected after a period of potential contact with sick cattle in March 1966.

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Poliomyelitis in the Netherlands before and after vaccination with inactivated poliovaccine

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INTRODUCTION

In most countries, where poliomyelitis was a major problem about 15 years ago, the disease has been reduced to negligible proportions by vaccination. Within a short period two effective vaccines became available, the live attenuated virus (o.p.v.) only a few years later than the inactivated poliovaccine (i.p.v.). The former vaccine is used nowadays in most parts of the world because of the ease of oral administration, the effective individual protection offered and the strong interference with the natural spread of the poliovirus. The latter type of vaccine, which must be administered parenterally, provides the immunized with a protection which as a rule equals that produced by the live vaccine. Depending on the amount of antigen it may even provoke a higher level of antibody (Henry *et al.* 1966). There are many observations (Henry *et al.* 1966; Dick *et al.* 1961) which show that i.p.v. interferes with alimentary infection but to a less extent than the live avirulent viruses.

The administration of i.p.v. can be simplified by combining it with diphtheria, tetanus and pertussis antigens, as a quadruple vaccine, which can easily be incorporated into a nationwide infant immunization schedule. Inactivated poliovaccine either alone or in such a combined vaccine has been used in the Netherlands since 1957 in the Government sponsored vaccination programme, which started in the autumn of 1957.

We decided to compare the poliomyelitis morbidity before and after vaccination began, in order to determine the degree of protection conferred on individuals and the community by this i.p.v. vaccination programme.

The figures for the incidence of poliomyelitis in the period 1924–65 were derived from reports of the Office of the Chief Medical Officer, as were the details, vaccination history, virus isolations etc., of the poliomyelitis cases occurring in the 1958–65 period. The number of vaccinated subjects in each age group was calculated from the acceptance rates taken from reports of the Office of the Chief Medical Officer and from population statistics (C.B.S. 1963, 1953–65). In this way the morbidity rates for vaccinated and non-vaccinated persons in each age group, and the percentage of morbidity reductions, were calculated.

METHODS AND MATERIALS

Vaccines

At first poliovaccine was imported from the U.S.A. (1957–59) and Belgium (1958–61). From 1961 formalin-inactivated poliovaccine produced in the Netherlands by the Rijks Instituut voor de Volksgezondheid (National Institute of Public Health) was used.

The Dutch formalin-inactivated poliovaccine is prepared from the Mahoney type 1, M.E.F.1 type 2 and Saukett type 3 strains of poliovirus. The potency of the vaccine lots was determined in monkeys according to the regulations of the U.S. National Institute of Health and is shown in Fig. 1. From 1962 the poliovaccine

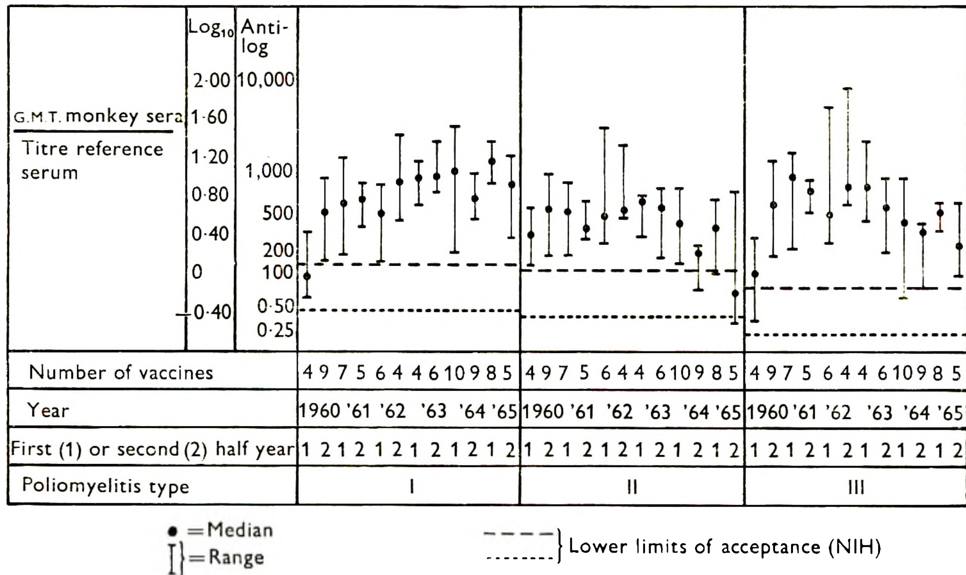

Fig. 1. Monkey potency test of poliomyelitis vaccine (NIH-test). Results of 77 lots produced from 1960 to 1965.

has been incorporated with diphtheria and tetanus toxoids and killed *Bordetella pertussis*. This quadruple vaccine contains 15 Lf diphtheria toxoid, 5 Lf tetanus toxoid and 16×10^9 *B. pertussis* organisms in a 1 ml. dose, and 1·5 mg. aluminium phosphate per dose is added as an adjuvant.

Vaccine schedules

In the early years two primary injections of plain poliovaccine given 1 month apart were followed by a booster dose about 6 months later. In the 3 years after the vaccination campaigns began late in 1957, all children born between 1945 and 1958 were offered the vaccine.

In 1960 the schedule was altered (Brandwijk *et al.* 1961). The children born in that year and later were given three primary injections at monthly intervals and a booster about 6 months later.

From 1962 onwards diphtheria, tetanus, pertussis and poliomyelitis were com-

bined in one vaccine. A primary series of three injections was given at the age of 3, 4 and 5 months respectively, followed by a booster injection at 11 months of age.

In 1964 and 1965 children born in the years 1952–9 were revaccinated with one dose of diphtheria–tetanus–poliomyelitis vaccine. Those born in 1960 and 1961 were offered revaccination in 1965 with either diphtheria–tetanus–poliomyelitis vaccine or diphtheria–tetanus–pertussis–poliomyelitis vaccine. From that year on this procedure became routine for revaccination at the age of 4–5 years against these diseases.

RESULTS

Incidence of poliomyelitis in the Netherlands

Since 1924, when poliomyelitis became notifiable, the disease has developed as is shown in Fig. 2. A distinction between paralytic and non-paralytic cases was made after 1951. In the 34 years from 1924 to 1957 the average annual number of

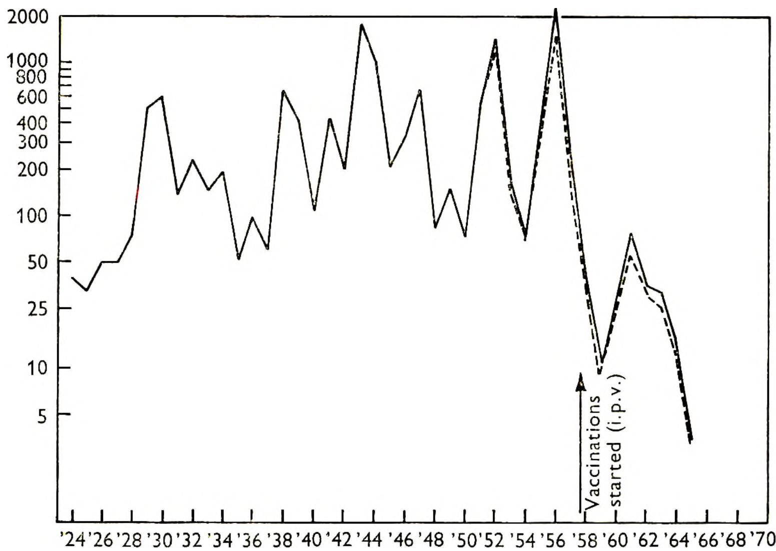

Fig. 2. Poliomyelitis anterior acuta. Notified cases in the Netherlands, 1924–65. —, All cases (paralytic and non-paralytic); - - -, paralytic cases.

all cases was 419 and the average morbidity rate per 100,000 inhabitants was 4·5. In the eight years from 1958 to 1965, covering the period in which polio vaccination became established, the average annual number of all cases was 31 and the morbidity rate 0·27 per 100,000, a reduction in morbidity of 94 %.

The lowest number of cases for any 8-year period between 1924 and 1957 was 1491 (1924–31), whereas 249 cases were reported between 1958 and 1965.

Table 1 shows the age distribution of poliomyelitis from 1950 to 1965. It is apparent that those most affected were the 1–4-year-old children, closely followed by those under 1 year of age. The 5–9-year children, particularly those of age 5 and 6, also showed a high rate. In the older age groups morbidity was less.

Effect of polio vaccinations

We compared the poliomyelitis morbidity in two 4-year periods (1950–3, 1954-7) before vaccinations began and in the 8 years after. Both 4-year periods included a pre-epidemic year (1951 and 1955), an epidemic (1952 and 1956) and a post-epidemic one (1953 and 1957).

Table 1. *Age distribution of poliomyelitis cases before (1950–3, 1954–7) and after (1958–65) vaccinations began*

Age (years)	1950–3 All cases	1950–3 %	1954–7 Paralytic cases	1954–7 %	1958–65 Paralytic cases	1958–65 %
< 1	167	6·6	183	7·4	16	7·5
1–4	1314	52·0	1324	53·7	89	42·0
5–9	490	19·4	587	23·8	53	25·0
10–14	160	6·3	90	3·7	19	9·0
15–19	99	3·9	54	2·2	6	2·8
⩾ 20	295	11·8	227	9·2	29	13·7
All	2525	100·0	2465	100·0	212	100·0

Table 2. *Reduction of poliomyelitis morbidity rates in different age groups before (1950–3 and 1954–7) and after (1958–65) vaccinations began*

Age (years)	Morbidity rate per 100,000: 1950–3 (all cases)	1954–7 (paralytic cases)	1958–5 (paralytic cases)	1958–65 % reduction compared with 1950–3	1954–7
< 1	18·5	20·3	0·84	95·5	95·9
1–4	35·3	37·3	1·20	96·6	96·8
5–9	11·3	12·9	0·59	94·8	95·4
10–14	4·7	2·2	0·21	95·5	90·5
15–19	3·1	1·6	0·07	97·7	95·6
⩾ 20	1·1	0·8	0·05	95·4	93·7
All	6·1	5·6	0·23	96·3	95·9

Table 2 shows the morbidity by age in the three periods and the reduction in morbidity rate expressed as a percentage. The over-all reduction is about 96 % and there are only small differences between age groups. The low incidence in the older age groups may reflect the influence of the epidemics in 1952 and 1956. Despite the vaccine being confined to those under 15 years there was no increase in morbidity in the older age groups.

From Table 3 the influence of the number of injections of poliovaccine can be seen. Of the 212 paralytic cases notified in the post-vaccination period (1958–65) 207 were considered and five were omitted. In two of these five patients the vaccination history was unknown, two more were vaccinated in other countries and could not, therefore, be compared with those vaccinated in the Netherlands; and the fifth showed signs of the disease upon arrival in this country.

The morbidity rates in three groups (non-vaccinated, 1 or 2 doses and 3 or more doses), are compared for three age groups from 1 to 14 years. In those under 1 year of age a comparison is not meaningful, because the main part of the vaccination schedule is performed in this group. Few above the age of 15 were vaccinated over this 8-year period, since vaccination was offered only to those born in 1945 and later. From table 3 it can be seen that there is a major reduction in morbidity after 1 or 2 doses of vaccine. The reduction for the three age groups is 85·7, 83·5 and 88·2 respectively. After completion of the vaccination schedule with three or more injections the reduction in morbidity in the children of 1–4 years is 95·7 %, in those of 5–9 years, 93·4 % and in the 10–14-year-olds 89·7 %. As the 1–4-year-old children include the most recently vaccinated, this may account for the difference in the fall of morbidity.

Table 3. *Vaccination history and age distribution of* 207 *paralytic poliomyelitis cases* (1958–65); *reduction of morbidity rate by vaccination*

Age (years)	Non-vaccinated		1 or 2 doses		3 or more doses		% reduction in morbidity in vaccinated compared with non-vaccinated	
	Cases	Rate per 100,000	Cases	Rate per 100,000	Cases	Rate per 100,000	1 or 2 doses	3 or more doses
< 1	14	< 2·0	1	—	0	—	—	—
1–4	53	8·75	13	1·25	22	0·38	85·7	95·7
5–9	32	3·33	6	0·55	15	0·22	83·5	93·4
10–14	12	0·68	1	0·08	4	0·07	88·2	89·7
15–19	6	0·15	0	—	0	—	—	—
⩾ 20	28	0·05	0	—	0	—	—	—

Table 4. *Reduction of morbidity rate* (1958–65) *in relation to age and vaccination history*

Age (years)	Non-vaccinated: % reduction in morbidity compared with		3 or more doses: % reduction in morbidity compared with	
	1950–3	1954–7	1950–3	1954–7
< 1	> 89	> 90	—	—
1–4	75	77	98·9	99·0
5–9	71	74	98·1	98·3
10–14	86	69	98·5	96·8
15–19	95	91	—	—
⩾ 20	95	94	—	—

Table 4 gives information about the degree of protection attained in non-vaccinated persons by the immunity of vaccinated individuals. For this purpose we compared the morbidity rates in such subjects over the years 1958–65 with the two preceding 4-year periods. We found a clear reduction in incidence of polio-

myelitis, but the reduction was different in the various age groups. In the children under 14 years it is about 70 %, but in those over 15 years it is more than 90 %. In the fully vaccinated subjects the morbidity fell by about 98–99 % compared with the unvaccinated.

Table 5. *Isolation of poliomyelitis virus in* 166 *cases of paralytic poliomyelitis* (1959–65)

Year	Poliomyelitis type I	II	III	Negative	Total tested
1959	0	0	1	7	8
1960	4	3	4	8	19
1961	55	0	5	7	67
1962	25	0	1	4	30
1963	17	0	2	8	27
1964	2	0	8	3	13
1965	0	0	2	0	2
Total	103	3	23	37	166

Table 6. *Virus isolations according to age and vaccination history in* 166 *patients* (1959–65)

Age group (years)	Vaccination history: 0 (−) Pos. No.	%	0 (−) Neg. No.	%	1 or 2 (±) Pos. No.	%	1 or 2 (±) Neg. No.	%	3 or more (+) Pos. No.	%	3 or more (+) Neg. No.	%
< 1	11	92	1	8	0	—	1	—	0	—	0	—
1–14	72	90	8	10	10	91	1	9	19	53	17	47
15–19	3	75	1	25	0	—	0	—	0	—	0	—
⩾ 20	14	64	8	36	0	—	0	—	0	—	0	—
All	100	85	18	15	10	83	2	17	19	53	17	47

Table 5 shows the results of poliovirus isolations in 166 paralytic poliomyelitis patients in the 7-year period 1959–65. Insufficient isolation results were available from the cases recorded in 1958. It can be seen that type II was isolated from polio patients in 1960 only. In 1961, 1962 and 1963 type I prevailed, but in 1964 and 1965 the picture changed and type III was more frequently isolated.

In Table 6 the results of virus isolations in the 166 patients in the period 1959–65 are shown by vaccination history and age. The percentage of those with positive virus isolations was reduced by about 40 % in the three or more times vaccinated compared with the non-vaccinated subjects. This probably indicates a relative decrease in virus excretion, but it may be due to paralytic disease not caused by poliovirus. Type III virus was relatively more frequently encountered than type I in vaccinated subjects. Thus in the 1–14-year-olds the ratio between the number of cases with virus isolations in the unvaccinated and vaccinated groups was 60:13 for type I and 9:6 for type III.

Because the coverage with poliovaccine in the Netherlands is slightly irregular, it appeared interesting to establish the relation between the morbidity of paralytic poliomyelitis in different municipalities and the acceptance rate. Figure 3 shows the results of our calculations. On the abscissa the municipalities are arranged in

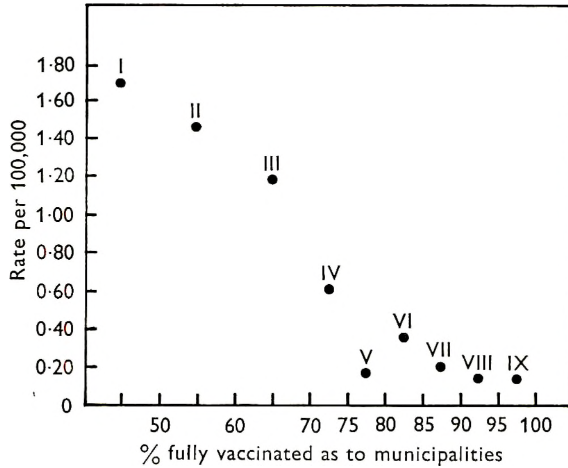

Fig. 3. 1958–65. Paralytic poliomyelitis. Relation between average yearly morbidity rate per 100,000 and the estimated percentage of children under 16 who had had three or more doses of polio vaccine, in different municipalities. I, 50 %; II, 51–60 %; III, 61–70 %; IV, 71–75 %; V, 76–80 %; VI, 81–85 %; VII, 86–90 %; VIII, 91–95 %; IX, 96–100 %.

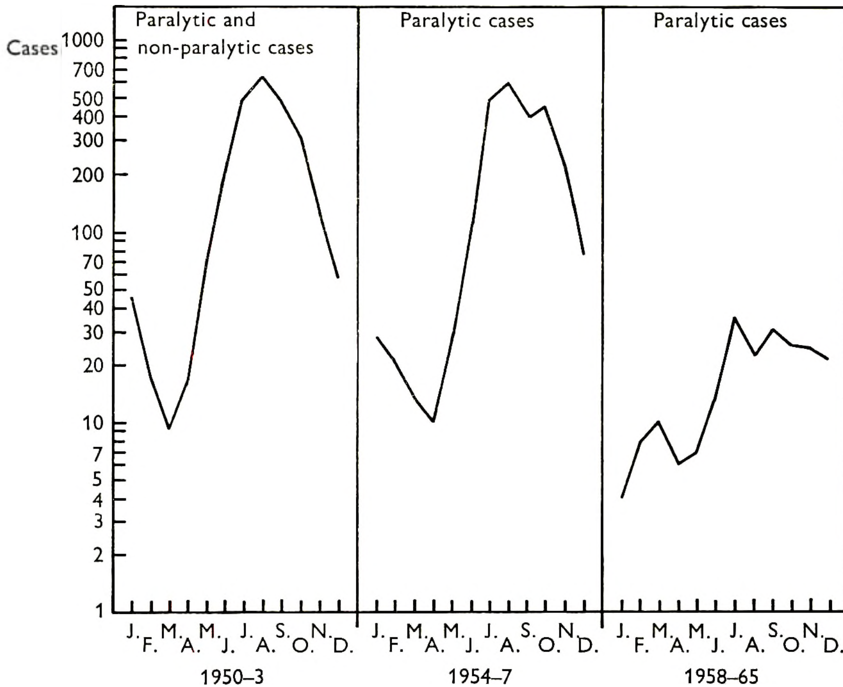

Fig. 4. Monthly distribution of poliomyelitis in the Netherlands in the periods 1950–3, 1954–7 and 1958–65.

nine groups according to the estimated percentage of three or more times vaccinated children under 16 years. This estimation was derived from the acceptance rate in September 1964 of the children born from 1959 to 1963.

The municipalities with the lowest percentages of fully vaccinated children showed the highest morbidity figures. The morbidity reached a relatively low, but constant level in the municipalities where the percentage of fully vaccinated was 75 % or more.

Finally Fig. 4 shows the monthly distribution of notified polio cases in the years 1950–3, 1954–7 and 1958–65. It is apparent that the seasonal curve is levelling off, despite the fact that in the post-vaccination period the highest number of cases still occurs in July.

DISCUSSION

From the beginning the vaccination campaign was directed particularly to the most vulnerable age groups, which are the most important disseminators of the infection, i.e. the children of 1–4-years and 5–9 years (Table 1). The decision to adopt the routine schedule, which starts at the age of 3 months and is completed before the end of the first year was a compromise. It was realized, that in the early months of life the response to the antigens would not be maximal (Perkins, Yetts & Gaisford, 1958, 1959), but in view of the higher risks associated with pertussis in very young infants it was thought that administration of this component could not be postponed. Moreover, for reasons of organization it was desirable to complete the schedule within the first year of life. By this procedure a high acceptance rate in those eligible for vaccination was reached. In 1964 for the 1–4-year-old children the overall acceptance rate for three or more doses was about 89 % and for those born from 1945 to 1959 it was 87–89 %.

The importance of the acceptance rate is shown in Fig. 3, from which it might be concluded that, under the conditions prevailing in the Netherlands, at least 75 % of the children, evenly distributed in a community, must be fully vaccinated to obtain a satisfactory reduction of poliomyelitis.

Against this background of good coverage, especially in the most susceptible age group (1–4 years) the results presented here must be considered. They make it clear that the reduction of paralytic poliomyelitis in the Netherlands is due to at least two factors; immunity in vaccinated people and a 'herd-immunity' as shown in Table 4. This latter phenomenon most probably is due to the decreased capacity of the vaccinated to spread poliovirus. The outcome of the virus isolations as presented in Table 6 and results from other investigators (Henry *et al.* 1966; Dick *et al.* 1961) support this explanation.

However, it might be useful in this respect to remark that in the adjacent countries, Belgium and the German Federal Republic, the circulation of wild polio viruses may have been reduced considerably by the use of o.p.v.

From Table 4 it can be seen that the highest level of polio reduction is reached in persons of 15 years and older. The decreased possibilities for shedding poliomyelitis virus by young children and the lower susceptibility with increasing age might have been responsible for these high figures.

Table 3 gives a good insight into the individual protection reached during the

period under study (1958–65), for all children considered in this table profited by the herd immunity, caused by the substantial vaccine coverage. The lower reduction rate for the fully vaccinated 10–14-year-old children could be an argument in favour of a revaccination between 4 and 10 years. The reduction rates, which are presented in Table 3, compare well with results published from other countries. For the U.S.A. a percentage of 91 in the age group 0–4 years and of 93 in the 5–14-year-old children was calculated (Langmuir, 1960). In Canada the 0–4-year group showed 87·4 % reduction, the 5–19-year group 97·1 % and the over-all percentage was 95·6 % (Kubryk, 1960). Data from a few outbreaks in Australia show the same general picture (Duxbury, Goulding & Graydon, 1963). In Sweden the reduction for all ages was 97·4 % (Olin, 1960). All these figures are based on the decrease of poliomyelitis in a comparatively short period after vaccination campaigns were completed. The reduction rates found by these investigators are higher than the first published results in the U.S.A. after the extensive 1954 field trial (Francis *et al.* 1955). The differences between the results mentioned above may be due to the quality of the vaccines, the vaccination schedule, the age groups covered and the acceptance rates.

The best estimate for the over-all protection against paralytic poliomyelitis in fully vaccinated children in the Netherlands can be made from the last two columns of Table 4. The morbidity has fallen by 97–99 % with slight differences for the age groups considered. These children have an individual protection and are further protected by the 'herd' immunity mentioned.

Finally, it may be of interest to compare the results of vaccinations in the Netherlands and in the four Scandinavian countries Denmark, Norway, Sweden and Finland. In Denmark o.p.v. type I was only used in a mass-campaign in 1963. For the rest i.p.v. was offered in these four countries.

In the Netherlands o.p.v. was used twice on a small scale to suppress local outbreaks. For this purpose in the summer of 1962 a few hundred doses of o.p.v. type I were used in Amsterdam and in December 1963 a few thousand doses of o.p.v. of the same type went to the island Tholen in the province Zeeland, a pocket with low acceptance rate, where some polio cases occurred.

Considering the situation in the Scandinavian countries in 1962–3 (Gard, 1964) we see a reduction of 90–95 % in Norway. In Denmark and Finland there are still a few cases but the reduction is more than 94 % and in Sweden poliomyelitis has virtually disappeared (Gard, 1966).

In the Netherlands the favourable picture which is so evident in Sweden is developing more slowly. After the rise in 1961, the number of paralytic cases steadily decreased to 3 in 1965, of which one was imported from Nigeria. Unfortunately 1966 brought a slight rise to 10 paralytic cases, mainly occuring in a region badly covered by vaccination. The different results in the Netherlands and Sweden may be due to several factors: population density, family size, housing conditions, pattern of coverage by vaccine, vaccination schedule and vaccine quality. The first three conditions are more favourable for virus dissemination in the Netherlands. Probably in Sweden the regions with a low acceptance rate are lacking. No comparison of vaccine potency has been made.

From the figures presented here, we may conclude that with inactivated polio-vaccine a good protection can be obtained provided that a vaccine of good quality is used and a high rate of acceptance is reached.

SUMMARY

Formalin-inactivated polio vaccine has been used in the Netherlands since 1957. Within 3 years all children born in 1945 and later were offered vaccine. In the first years poliovaccine from different manufacturers were used. In 1961 a vaccine produced in the Netherlands by the 'Rijks Instituut voor de Volksgezondheid' (National Institute of Public Health) became available and in 1962 the poliomyelitis components were incorporated in a quadruple vaccine, which contains 15 Lf diphtheria, 5 Lf tetanus toxoid and 16×10^9 *Bordetella pertussis* organisms in addition to the three polio components. As an adjuvant this quadruple vaccine contains 1·5 mg. aluminium phosphate per 1 ml. dose. For infants the schedule became three doses at ages 3, 4 and 5 months respectively, followed by a booster dose at the end of the first year. The over-all acceptance rate can be estimated at almost 90 % of those eligible, but there were pockets in the population with lower rates.

Since 1924 poliomyelitis has been a notifiable disease. During and after the last World War major epidemics occurred.

The poliomyelitis morbidity rates in the 8 post-vaccination years 1958–65 were compared with those from two preceding 4-year periods 1950–3 and 1954–7. In the 1–4-year-old children, who presented the most vulnerable age group, paralytic poliomyelitis was reduced by about 97 % and in the other age groups this percentage was slightly less. The over-all reduction was 96 %. Comparison of the morbidity rates of non-vaccinated persons in 1958–65 with the rates from 1950–3 and 1954–7 gave an impression of the extent of the herd immunity. These rates were reduced 70–80 % in the children under 15 years and 90–95 % in those over this age. The individual protection given to the vaccinated was calculated from the morbidity rates in non-vaccinated, incompletely and fully vaccinated persons in the 1958–65 period. The reduction of morbidity was 90–95 % for children of 1–14 years who got three doses or more and about 85 % for those who had only 1 or 2 doses. Fully vaccinated children, who are profiting from both individual protection and herd immunity, showed a reduction of 97–99 %.

From the data presented it is concluded that vaccination with inactivated poliomyelitis vaccine can provide very effective protection for the individual and the community provided that a vaccine of good quality is used and the rate of acceptance is 75 % or better.

I wish to thank Drs B. V. Bekker and J. Bijkerk of the Office of the Chief Medical Officer and Dra. Ch. A. Hannik for providing me with data about polio cases, virus isolations and vaccine acceptance rates, and Dr A. J. Beale for reading the manuscript and giving useful suggestions.

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Nasal and skin carriage of *Staphylococcus aureus* by patients undergoing surgical operation

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It is generally accepted that the nose is the most frequent site of carriage of *Staphylococcus aureus* in man, and regular swabbing of the nose has been used in many epidemiological investigations as a means of sampling the staphylococci carried by hospital patients and staff. It is common experience, however, that no source can be found among the nasal carriers present in the ward for a substantial minority of the strains acquired by patients or present in the ward air (see, for example, Shooter *et al.* 1963; Lidwell *et al.* 1966). Persons with negative nasal swabs occasionally carry *Staph. aureus* elsewhere on the surface of the body and there is evidence that some of them—especially those carrying the organism in the perineal region—may be of particular significance as dispersers of the organism into the environment (Hare & Ridley, 1958; Ridley, 1959; Solberg, 1965). Bøe *et al.* (1964) examined 3508 patients on admission to hospital, and found that 12·8 % carried *Staph. aureus* on the perineal skin; 4 % had positive perineal but negative nasal swabs.

We took the opportunity, while carrying out an investigation of the aerial dispersion of *Staph. aureus* in the operating theatre at the West Herts Hospital, Hemel Hempstead (Lidwell, Polakoff & Richards, 1967) to obtain cultures from several sites on the body-surface of patients immediately before operation.

METHODS

The investigation was carried out on 361 patients of two general surgeons, and included nearly all of those operated upon in the course of 124 morning sessions during 18 months. The patients were bathed and shaved in the ward, but received no other pre-operative skin treatment there. A freshly laundered pack for each patient was delivered to the ward on the morning of the operation; it contained a canvas stretcher, a drawsheet, a cotton cellular blanket, cotton leggings, and a gown. Patients were dressed in gown and leggings about an hour before the operation. They were anaesthetized in the theatre annexe while on the stretcher on which they had been brought from the ward. They were then wheeled into the theatre and the blanket was removed.

Swabs for bacteriological examination were collected from the nose, perineum, axilla and hand in the theatre while the patient was being prepared for operation. A cotton-wool swab, which had first been moistened with nutrient broth, was used

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to sample both anterior nares, six circular sweeping motions being made in each. The swab was then placed in a test-tube containing 2 ml. broth for transport to the laboratory.

Other skin sites were sampled with a large swab made by wrapping several layers of 1 in. wide cotton gauze bandage round the end of a 6 in. × $\frac{3}{4}$ in. wooden throat spatula and tucking in the end. This swab was moistened with broth and then rubbed to-and-fro six times on the chosen surface. The gauze end was then pushed off with a separate sterile applicator into a screw-capped jar containing 10 ml. broth.

The perineal swab was taken from the skin area extending from 1 in. in front of the anus to the base of the penis in the male, and to the lower border of the symphysis pubis in the female. The axillary swab was taken from the whole of the left axilla, and the hand swab from the dorsum of the right hand and wrist.

All swabs were transported to the laboratory at the end of the operating session and were at ambient temperature in the meantime. The interval between the collection of the swabs and the inoculation of primary plates was usually between $1\frac{1}{2}$ and 4 hr., and did not exceed 5 hr.

The following bacteriological media were used: 7 % horse-blood agar and nutrient agar with 7 % added sodium chloride (salt agar). Inoculated plates were examined after 1 and 2 days' incubation at 37° C. Nasal swabs were rubbed on a separate blood-agar plate and then returned to the tube of broth, which was incubated overnight and re-plated on salt agar. For all other swabs, one loopful of broth from the jar was inoculated on a blood-agar plate and another on a salt-agar plate. The broth was also incubated overnight and then subcultured on a salt-agar plate.

One representative of each colonial type on each plate which resembled *Staph. aureus* was subcultured and a coagulase test was performed. Each coagulase-positive culture was phage-typed and tested for resistance to penicillin and tetracycline by streaking up to filter-paper strips impregnated with antibiotic.

Two cultures of *Staph. aureus* isolated from the same patient were considered to belong to distinct strains if their phage-typing patterns differed by two or more strong reactions (Williams & Rippon, 1952) or if their sensitivity to one or both antibiotics was different.

RESULTS

A swab was collected from each of the four sites (nose, perineum, axilla and hand) in 361 patients. Table 1 shows the number and percentage of swabs from each site in which *Staph. aureus* was found by direct plating (+ + +) or only by enrichment (+), and the total positive by both methods. The nasal carrier-rate was 40 %, and seven-eighths of the isolations were made by direct plating; 12 % of the perineal swabs were positive, nearly half of them by direct plating; isolations from the axilla (7 %) and from the hand (24 %) were in most cases made only by enrichment culture. Heavy carriage was thus characteristic of the nose and, to a lesser extent, of the perineum, but carriage in the axilla and on the hand was usually scanty.

In Table 2, the distribution of *Staph. aureus* cultures with different patterns of sensitivity to penicillin and tetracycline is shown. The cultures were divided into

the following groups: S: sensitive to penicillin and to tetracycline; P: resistant to penicillin but sensitive to tetracycline; R: resistant to tetracycline and, with a few exceptions, also to penicillin.

The total of the three columns S, P and R exceeds the total number of positive swabs because two or more distinct strains were isolated from some of the swabs. The figures in the three columns represent the number and percentage of swabs from each source which contained staphylococci in the corresponding resistance-category.

Table 1. *Isolation of* Staphylococcus aureus *by direct plating and by enrichment culture from swabs of the nose, perineum, axilla and hand of* 361 *patients*

(+ + +, by direct plating; +, only by enrichment. Percentages in parentheses.)

	Staph. aureus isolated		
	+ + +	+	Total
Nose	128 (35)	18 (5)	146 (40)
Perineum	20 (6)	25 (7)	45 (12)
Axilla	2 (< 1)	22 (6)	24 (7)
Hand	13 (4)	74 (20)	87 (24)

Table 2. *Antibiotic resistance of* Staphylococcus aureus *strains from the nose, perineum, axilla and hand*

(S, sensitive to penicillin and tetracycline; P, resistant to penicillin, sensitive to tetracycline; R, resistant to tetracycline. Percentages in parentheses.)

	Total swabs positive	No. (and percentage) of swabs containing organisms with the following sensitivities		
		S	P	R
Nose	146 (40)	92 (25)	48 (13)	15 (4)
Perineum	45 (12)	27 (7)	11 (3)	8 (2)
Axilla	24 (7)	10 (3)	10 (3)	4 (1)
Hand	87 (24)	44 (12)	33 (9)	12 (2)

Although the carrier-rate for resistant organisms in the nose was higher than that at other sites, the proportion of the organisms carried that were resistant was somewhat greater at the other sites than in the nose. Thus, only 15 of 146 cultures isolated from the nose (10 %), but 24 of 156 from other sites (15 %) were 'multiple resistant' (R).

The age- and sex-distribution of carriage at each site is summarized in Table 3, which shows the percentage of swabs positive on direct culture, with the total percentage of positive swabs in parentheses. The nasal carrier-rate was, as expected, higher in the young patients. Carriage on the skin of the axilla and the hand was also rather more common in the young than in the old. Perineal carriage, on the other hand, appeared to occur with equal frequency at all ages. There was no significant difference between the carrier-rates in males and females at any of the sites, either in the totals or within the age groups.

Table 4 shows the number of positive cultures from the perineum, axilla and hand in relation to the nasal carrier-state. Carriage at all sites was significantly related to the presence of *Staph. aureus* in the nose. Among the 146 patients with positive nose-swabs, 36 (25 %) were perineal carriers, 20 (14 %) were axillary carriers, and 68 (47 %) were hand-carriers. The corresponding figures for the 215

Table 3. *Relation of age and sex to carriage-rates for* Staphylococcus aureus

(Percentages of swabs positive by direct plating, with total percentage positive (direct + enrichment) in parentheses.)

Age (years)	No. examined	Percentage positive: Nose	Perineum	Axilla	Hand
0–9	34	50 (50)	6 (9)	0 (12)	3 (35)
10–19	26	62 (69)	12 (19)	5 (15)	15 (54)
20–29	20	50 (55)	5 (15)	5 (10)	5 (15)
30–39	51	31 (33)	2 (10)	0 (4)	2 (16)
40–49	74	30 (35)	5 (12)	0 (7)	3 (26)
50–59	66	30 (35)	5 (17)	0 (8)	3 (21)
60+	90	30 (38)	7 (10)	0 (2)	2 (19)
Sex					
M	219	37 (42)	6 (12)	< 1 (6)	5 (28)
F	142	32 (39)	5 (13)	1 (8)	1 (18)
Total	361	35 (40)	6 (12)	1 (7)	4 (24)

Table 4. *Number of perineal, hand, and axillary cultures positive for* Staphylococcus aureus *in nasal carriers and non-carriers*

(+ + +, positive on direct plating; +, positive only on enrichment.)

	Nose swab: + + +	+	Negative
All nose swabs	128	18	215
Perineum			
+ + +	12	3	5
+	19	2	4
Negative	97	13	206
Axilla			
+ + +	2	0	0
+	17	1	4
Negative	109	17	235
Hand			
+ + +	11	0	2
+	50	7	17
Negative	68	11	195

patients with negative nose swabs were: perineal carriers 9 (4 %), axillary carriers 4 (2 %), hand carriers 19 (9 %). Thus, though the presence of staphylococci on the skin might in many cases be attributable to contamination with nasal secretion, in other cases it was independent of nasal carriage.

The isolation of *Staph. aureus* from the skin by the enrichment method only

might be a consequence of recent contamination from an extraneous source, but the presence of the organism in numbers sufficient to be detected by direct plating probably indicates that it has colonized the body surface, particularly if the nose-swab is negative, or is positive only on enrichment. It is therefore of interest that eight of the 20 heavy perineal carriers (+ + +) were patients with few *Staph. aureus* in the nose (+), or with negative nose swabs. The two heavy axillary carriers, and all but two of the 13 patients with heavy hand carriage, had nose swabs that were positive for *Staph. aureus* on direct culture (+ + +).

Table 5. *Relative frequency of independent carriage of* Staphylococcus aureus *on the perineum, axilla and hand*

(+ + +, positive on direct plating; +, positive only on enrichment. Strains isolated from each site are divided as follows: line 1—same strain + + + in nose; line 2—nasal swab negative, contained a different strain, or the same strain +.)

		No. of strains			
		+ + +		+	
Antibiotic resistance		S + P	R	S + P	R
All nasal swabs		122	12	18	3
Perineum	1	10	1	23	3
	2	5	4	0	0
Axilla	1	2	0	11	2
	2	0	0	7	2
Hand	1	8	2	38	1
	2	3	0	26	9

The extent of independent carriage in the perineum, axilla and hand was examined further in Table 5, in which the strains of *Staph. aureus* isolated from these three sites were divided into two groups of: (1) those in which the same strain was isolated from the nose by direct plating; and (2) those in which the corresponding nasal swab was negative, was positive for a different strain of *Staph. aureus*, or yielded the same strain in such small numbers that it could be detected only by enrichment culture.

The cultures from the three sites were subdivided further into those isolated on direct plating (+ + +) and those recovered only after enrichment (+), and because it appeared that there was an excess of antibiotic-resistant strains among the cultures isolated from the skin, also into those sensitive to both antibiotics or resistant only to penicillin (S + P) and those resistant to tetracycline (R).

There was clear evidence of a group of patients carrying *Staph. aureus* independently on the perineal skin. Nearly half of the heavy perineal carriers (9 out of 20) were not heavy carriers of the same strain in the nose. On the other hand, all 26 of the patients whose perineal swabs yielded a scanty growth of *Staph. aureus* were heavy carriers of the same organism in the nose. The distribution of antibiotic resistance in the strains that were carried independently in the perineum was also quite unlike that in the strains present in both nose and perineum, or indeed in the

nose swabs of all patients. Of cultures isolated by direct plating (+ + +), four of the nine from independent perineal carriers, but only one of 11 from perineal carriers who were also nose carriers of the same strain, and 12 of 134 from all nasal carriers were tetracycline resistant. This suggested that 'multiple-resistant' hospital staphylococci were more likely than other strains to cause independent perineal carriage.

When, however, axillary and hand carriage were examined in the same way, the pattern was quite different. The only two heavy axillary carriers, and 10 of the 13 heavy hand carriers also carried the same strain prolifically in the nose. In addition, the distribution of antibiotic resistance among strains carried on hands and axilla was not significantly different from that found among nasal strains, and none of the three strains carried heavily and independently on the hands was tetracycline resistant.

The nine patients who were heavy, independent perineal carriers included four males and five females. The organisms from two of the males and from two of the females were tetracycline resistant. Patients who were independent perineal carriers of tetracycline-resistant organisms had been in hospital on average 15 days (respectively 5, 12, 16 and 27 days); those carrying sensitive organisms had been admitted to hospital more recently (respectively 1, 1, 1, 3 and 6 days before operation).

DISCUSSION

Perineal carriage of *Staph. aureus* was less common among our patients, who had recently bathed and donned fresh clothing, than among the male medical students examined by Ridley (1959). He found the organism in perineal swabs from 13 of 40 students (32 %) who were examined only by a direct cultural method, but the clothes of the subjects were often heavily contaminated with staphylococci at the time of sampling. Our patients were examined both by direct and by enrichment sampling, and the total perineal carrier-rate was 12 %. This figure is similar to the one observed by Bøe *et al.* (1964) in Norwegian hospital patients who had been bathed and reclothed just before swabbing. The bacteriological methods used by these workers appear to have been of similar sensitivity to those used in the present investigation.

Skin carriage, whether in the perineal or axillary region or on the hand, was more frequent among nasal carriers of *Staph. aureus* than among non-carriers; but though hand and axillary carriage was usually scanty, half of the perineal carriers were heavy carriers in whom the organism could be detected by direct cultural methods. The isolation of *Staph. aureus* by direct plating indicated a recovery of at least 1000 colony-forming units from a skin swab.

Our main interest was in the frequency with which patients with negative nose-swabs might be unsuspected sources of *Staph. aureus* infection. A similar proportion of our patients (4 %) as of those examined in Norway (Bøe, *et al.* 1964) had a positive perineal and a negative nasal swab when both direct and enrichment cultures were performed.

We examined the incidence of independent carriage of *Staph. aureus* in the

perineal region, and for comparison also in the axilla and on the hand. An independent carrier at one of these sites was defined as a person whose skin swab was positive for *Staph. aureus*, but whose nasal swab did not yield the same strain on direct plating, and would therefore not have been detected in routine epidemiological investigations. Independent skin carriage was considered to be heavy when the organism was isolated by the direct plating method.

Whenever *Staph. aureus* was found in small numbers in the perineal swab, the same strain was isolated by direct plating from the nasal swab. Among heavy perineal carriers, however, nearly half (9 of 20) were independent carriers. These were probably people in whom the perineum was the primary site of staphylococcal multiplication. Examination of perineal swabs by direct plating thus revealed that over 2 % of the patients were potential sources of *Staph. aureus* which would not have been detected by routine nasal swabbing, and increased the number of carriers of *Staph. aureus* by 5 %. C. O. Solberg states (personal communication, 1967) that, among 100 persistent carriers examined by a standardized test (Solberg 1965), there were 16 heavy dispersers, and that six of them (38 %) were heavy carriers of the same strain on the perineal skin, whose nose swabs however yielded few staphylococci or were negative. This suggests that examination of nasal swabs only might leave undetected up to one-third of the more profuse aerial dispersers of *Staph. aureus*.

The distribution of antibiotic resistance among strains carried independently on the skin of the perineum was different from that found in perineal carriers who were also nasal carriers of the same strain, and in all nasal swabs. The proportion of tetracycline-resistant strains (R) isolated from heavy, independent perineal carriers (4 of 9) was significantly higher ($\chi^2 = 6{\cdot}9$) than that among all heavy nasal carriers (12 of 134). Independent carriage in the axilla and on the hand was nearly always scanty, and the distribution of antibiotic resistance among the strains isolated was not significantly different from that found in strains isolated from the nose.

Most of the patients examined in the present investigation had recently been admitted to hospital, and the carrier-rate for tetracycline-resistant *Staph. aureus* was low (e.g. 4 % in the nasal swabs). The independent perineal carriers of tetracycline-resistant staphylococci had been in hospital considerably longer on average than the rest of the patients. To confirm our finding that there was an excess of antibiotic-resistant 'hospital' strains among staphylococci carried independently on the perineal skin, and to define the conditions under which these organisms were acquired, a much larger investigation, in which nose and perineal swabs were examined serially, would be necessary.

Antibiotic-resistant staphylococci might reach the perineum in the faeces. Perineal carriers are not usually heavy faecal carriers of *Staph. aureus* (Solberg, 1965), but multiplication of the organism in the bowel contents earlier in the stay in hospital cannot be excluded. Alternatively, the organism might be conveyed directly to the perineum in the course of procedures carried out in the ward before operation. It is noteworthy that all four of the independent perineal carriers of 'hospital' staphylococci were among the 24 % of the patients who underwent operations for disease of the genito-urinary system or the lower bowel (one each:

carcinoma of the bladder, carcinoma of the rectum, urethral stricture, hydronephrosis).

The knowledge that some important sources of *Staph. aureus* cannot be detected by the examination of nasal swabs adds to the difficulty of controlling staphylococcal infection in hospital wards. Repeated perineal swabbing is an unpopular measure. There is need for a simple and reliable means of sampling the personal environment of patients for *Staph. aureus* in a way which would reveal the independent perineal carriers.

SUMMARY

Nasal, perineal, axillary and hand swabs collected from 361 patients immediately before operation were examined for *Staphylococcus aureus*.

The organism was isolated more often from all three skin sites in nasal carriers of *Staph. aureus* than in non-carriers.

Twelve per cent of the patients, and 4 % of those with negative nose swabs were perineal carriers. Two per cent were heavy perineal carriers of *Staph. aureus* strains which could not be isolated by direct culture from a nasal swab. Staphylococcal strains from these heavy, independent, perineal carriers were more often resistant to tetracycline than were strains from nasal carriers.

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Carriage of *Staphylococcus aureus* in random samples of a normal population*

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INTRODUCTION

Although a great many studies have been carried out on the nose and throat carriage of staphylococci, few investigators have had the opportunity of studying an unselected population. In a comprehensive review of staphylococcus carriage, Williams (1963) lists 15 published investigations on non-hospital populations; of these 10 were on populations selected by age or occupation, e.g. medical students, undergraduates or armed forces personnel, two were on patients attending their general practitioners or out-patient departments and three were on persons selected in an unspecified manner from the general population.

During the course of a survey on the incidence and prevalence of rheumatic fever and rheumatoid arthritis under the direction of one of us (H. A. V.), the opportunity arose of studying staphylococcal carriage in a randomly selected sample of a population. This paper describes the findings.

The population sampled was that of three villages about 3 km. apart in a rural environment but close to the industrial town of Eindhoven in the southern part of the Netherlands. As the total population of the three villages was of the order of 15,000 people, it was not possible to sample all individuals. In the Netherlands, however, a complete register of all persons residing in an area is maintained at the municipal offices; from this register it was possible, by the use of random number tables, to select true random samples from the population. The municipal register gives not merely the names but also the addresses, ages, family size, religion and other details. It was therefore possible to contact each person and to send them a letter explaining the nature of the survey, and subsequently to send a card asking them to attend at a local clinic at a specific time.

The persons to be seen each month were drawn as separate random samples, rejecting from the sample population only those drawn previously and those under 6 years of age. Thus each month's respondents constituted a separate random sample of the population. As all respondents were seen twice, each monthly sample consisted of about 120 persons seen for the first time, plus 120 seen for the second time. The initial response to the invitation cards was of the order of 75 % and by

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making home visits the completion rate was brought to about 95 %. In addition to the sample population the entire inhabitants of two old people's homes were examined on one occasion only, in a separate study. Some of these people were, of course, included in the random samples.

METHODS

Cotton-tipped swabs were used to obtain the samples; dry swabs were used for throat samples, but swabs moistened with broth were used on the forearm and for nasal swabs. The fingers were sampled by rubbing the finger tips directly on the agar plate. All cultures were made on 5 % sheep blood agar. Since the primary interest in the throat swabs lay in the isolation of β-haemolytic streptococci, these swabs were inoculated on agar and then into blood broth for enrichment and the plates incubated anaerobically; all other plates were incubated aerobically. Antibiotic sensitivity was determined using the disk method. Most of the phage-typing was very kindly done for us at the Central Public Health Laboratory, England, by Dr M. T. Parker.

In assessing the results, any swab giving no growth has been omitted on the grounds of faulty technique. Only respondents from whom at least one full set of data was obtained have been included in the analyses.

Respondents were asked the following questions:

(1) Have you a skin infection at this moment; if so have you been to your doctor about it; if 'yes', which doctor?

(2) Have you been in hospital in the last 6 months?

(3) Has anyone at home been in hospital in the last 6 months?

(4) Have you been to an out-patient department in the last 6 months?

(5) Has anyone at home been to an out-patient department in the last 6 months?

(6) Have you had penicillin in the last 6 months?

Respondents were allowed to answer 'yes' or 'no' or 'uncertain'. In analysing these results 'uncertain' has been counted as 'no'.

The word staphylococcus is used throughout this paper to mean the coagulase positive *Staphylococcus aureus*.

RESULTS

Nose and throat carriage

In no part of the survey was any clear difference in carriage seen between the male and female respondents and the results have accordingly been pooled. Table 1 shows the age composition of the population, the sample population drawn for the months November to May and the percentage of persons from whom adequate data were obtained. The total number of respondents seen in this survey was approximately 7·5 % of the population.

The distribution of staphylococcus carriage by age is shown in Table 2. In general there was a decrease in the rate of carriage of both nasal and throat staphylococci with increasing age; this trend was also apparent when the figures were

analysed month by month. The figures for the old people's homes are slightly higher than might have been expected on the basis of the normal population, but some cross-infection was obvious in one of the homes, five of the people carrying the same staphylococcus. Although the age trend was quite consistent for each of the three villages, there were differences between the villages in the carriage of penicillin resistant staphylococci and it is possible that these reflect differences in

Table 1. *Age distribution of the populations*

Age group	Total population	Sample population in percentages	Percentage from whom adequate data were obtained
6–9	1445	7·1	89
10–14	1593	8·0	95
15–19	1303	8·4	94
20–29	2184	8·0	94
30–39	1734	7·4	99
40–49	1155	8·9	92
50–59	914	8·3	88
60–69	618	6·3	88
70+	555	6·7	92
Total	11501	7·8	93
Old people's homes			
65–69	27	100	93
70+	163	100	96

Table 2. *Distribution of staphylococcus carriage by age*

(These results are based on the first visit only.)

Age group	Total no.	Percentage carriers		
		Nose carriers	Throat carriers	Nose and throat carriers†
6–9	92	33	14	7
10–14	121	42	13	7
15–19	103	37	13	4
20–29	166	27	13	6
30–39	127	29	6	2
40–49	95	28	8	4
50–59	67	25	3	2
60–69	34	0	3	0
70+	34	15	3	0
Old people's homes				
65–69	25	28*	4	4
70+	157	18*	3	1

* Some 'cross-infection' was obvious in one of the homes.

† Overall a total of 51 pairs of nose and throat staphylococci were available for comparison: in 36 (71 %) of the pairs the strains were identical on the basis of phage-typing.

the prescribing patterns of the local general practitioners. The differences are not statistically significant however (10 % > P > 5 %, χ^2 = 3·6).

Because each monthly sample was a separate random sample from the population, it was possible to use the figures as a measure of the intersample variation (Table 3). There was considerable variation from month to month in nasal carriage but these monthly variations were no greater than the variations between the sample of people seen for the first time each month and those seen for the second time each month, i.e. the differences within months were statistically as large as those between months (F = 1·08, n_1 = 5, n_2 = 6, P > 20 %). This would seem to rule out any possibility of seasonal variation, for differences between months should then be greater than those within months. The mean carrier rate for any one sample was 29 % with a standard deviation of 7 %.

Table 3. *Monthly variation in carriage rates*

	Respondents seen each month					
	For 1st time			For 2nd time		
		Percentage			Percentage	
Month	Total	Nasal carrier	Throat carrier	Total	Nasal carrier	Throat carrier
December 1964	124	26	19	116	33	11
January 1965	119	40	9	119	34	10
February 1965	117	19	9	117	30	9
March 1965	119	31	8	119	39	8
April 1965	118	30	7	118	21	9
May 1965	120	27	4	112	20	1

Because the respondents were seen twice, a comparison of carriage over a month was possible. The percentage of carriers at the first and second sample was the same (29%). The total nasal carrier rate, that is those who carried staphylococci at either one or both visits, was 41·2 % with a standard deviation of 6·3 %. No differences in frequency of acquisition or loss of the staphylococci were seen in respect of age or sex of the respondents; there was a suggestion that staphylococci resistant to penicillin were more 'mobile' than those sensitive to penicillin but the differences are not statistically significant.

Carriage on skin

On one occasion the mid-anterior forearm of 201 respondents was swabbed; nine of the swabs yielded *Staph. aureus* but only one of these yielded more than six colonies on the agar plate. Seven of the nine were nasal carriers and the other two were throat carriers. In a total of 476 respondents the fingers were sampled by rubbing on an agar plate. Thirteen per cent of the 120 nasal carriers yielded more than six colonies of staphylococci from the fingers compared with 4 % of the 356 non-carriers (P < 0·1 %, χ^2 = 18·1). Phage-typing showed that in 20 of the 21 pairs of nose and finger staphylococci the strains were the same, indicating that many of the staphylococci acquired on the hands were of nasal origin.

Results from the questionnaire

From the questionnaire the number of respondents having skin disease, penicillin therapy, etc., was compared with the total carrier rate, but although there was a suggestion that carriers of staphylococci had a higher skin disease rate, none of these differences is statistically significant using the χ^2 test (Table 4). However, respondents carrying penicillin-resistant staphylococci in the nose had skin diseases more frequently than those who were non-carriers. Thus 18% of the 67 nasal carriers of resistant staphylococci had skin disease compared with 7% of the 178 who carried a sensitive strain and 9% of the 587 non-carriers. The difference in the incidence of skin disease between the carriers of resistant and sensitive strains is significant between the 1 and 0·1% levels ($\chi^2 = 8{\cdot}5$).

Table 4. *Staphylococcus carriage in relation to questionnaire*

	Nose		Throat	
	Non-carriers	Carriers	Non-carriers	Carriers
	Percentage giving positive answer to questions			
(1) Presence of skin infection	9	10	9	13
(2) Hospitalization, Self	4	3	3	6
(3) Hospitalization, Family	15	12	14	16
(4) Outpatient visit, Self	12	11	12	13
(5) Outpatient visit, Family	25	28	27	24
(6) Penicillin therapy	4	4	4	1
Total number of persons from whom adequate data were obtained	587	245	750	82

Table 5. *Family carriage of staphylococci*

Secondary case	Index case: Non-carrier	Index case: Carrier	Total
Non-carrier	54	17	71
Carrier	23	19	42
Total	77	36	113

The index case was the first respondent seen in each pair.
The excess of double carriers is significant between the 5% and 2% levels.

Family analysis

In a random sample from a population where the family size is large, some families yield no respondents whilst other families contribute more than one member. This makes it possible to analyse carriage within families by taking the first family member as the index case, irrespective of age, etc., and the next member as the secondary case. As shown in Table 5 there was a significant excess of carriers in the families of carriers. This might be expected if the staphylococci carried were

the same in each of the pairs but this was not the case, for of the 19 pairs of double carriers, only seven pairs carried the same strain. On this basis one might postulate some genetic susceptibility to nasal carriage of staphylococci, for if simple environmental exposure were involved, i.e. if cross-infection occurred between the family members, one would expect the nasal strains to be the same in both respondents.

DISCUSSION

The fact that each of the monthly samples was a separate random sample from the population enabled an estimate to be made of the variation to be expected in the nasal carrier rate of a population. The various carrier rates ranged from 19 to 40 % with a mean of 29 % and a standard deviation of 7 %. On the basis of these figures derived from a rural population having only normal hospital contact one might predict that 'normal' carrier rates for nasal staphylococci derived from a single swabbing would lie within the range 15–43 % and this effectively covers the range given by Williams (1963). In a population with an age bias, such as undergraduates, we might expect a biased carrier rate, for some effect of age was apparent in these investigations. Clearly care is needed before describing a particular population as having an abnormally high or low carrier rate. When the results for the two swabbings are added together the carrier rate is higher (41 %) but there is still a considerable standard deviation (6 %) giving an 'expected' range of 29–53 %.

Analysis of carriage within families suggested that there might be some family predisposition to carry staphylococci. That this predisposition was not simply a reflexion of exposure to some common reservoir was suggested by the fact that in 12 of the 19 pairs of strains the staphylococci were of different phage types. Hoeksma & Winkler (1963) in a study of the nasal flora of 32 pairs of identical and 35 pairs of single sex, non-identical twins concluded that: 'The degree of similarity of the nasal flora of both partners of identical twins was much higher than for non-identical twins pointing definitely to genetic influences on the resistance of the nasal mucosa.' The present work supplements these observations and supports the view that genetic influence plays some part in determining nasal carriage of staphylococci.

It is perhaps surprising not to have been able to draw some positive conclusions on the influence of penicillin therapy and stay in hospital on the carrier rates of staphylococci. Patients discharged from hospital may retain their staphylococci for long periods (Noble *et al.* 1964) and other surveys have demonstrated clear effects of hospitalization or antibiotic therapy (Galbraith, 1960; McDonald *et al.* 1960). In this series spread of staphylococci from one person to another may have blurred the picture. An instance of spread was encountered in which two respondents carried an identical, typically 'hospital' staphylococcus (phage type 83A, resistant to penicillin, tetracycline, streptomycin and sulphonamides). One respondent had been a hospital inpatient but the other denied all hospital contact. The apparent anomaly was explained when it was discovered that the second respondent did the housework of the woman who had been in hospital; she had presumably acquired her nasal staphylococcus from her employer. Only 10 of the

respondents carried staphylococci resistant to tetracycline, but these ten people had not been in hospital or received penicillin therapy more often than had carriers of tetracycline-sensitive staphylococci.

SUMMARY

Nose, throat and finger carriage of *Staphylococcus aureus* was investigated in a series of random samples from a normal European population.

No evidence for a seasonal trend in carriage was found but the intersample variation between successive random samples was obtained. The mean nasal carrier rate was 29 % with a standard deviation of 7 %.

No association was found between nasal or throat carriage of staphylococci and stay in hospital or antibiotic therapy but respondents with penicillin-resistant staphylococci in the nose had skin infections more frequently than those with penicillin-sensitive strains.

Evidence was obtained for a family, perhaps genetic, 'predisposition' to carry staphylococci in the nose.

We wish to express our thanks to the people of Bladel, Reusel and Hapert who by generously taking part made this study possible.

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Classification of *Mycobacterium avium* and related opportunist mycobacteria met in England and Wales

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Amongst the opportunist (syn. anonymous) mycobacteria responsible for human infections, the most important and widely distributed are those designated 'group III; Nonphotochromogens' by Runyon (1959) and 'dysgonic nonchromogens' (groups 4 and 5) by Marks & Richards (1962). In Britain, the dysgonic nonchromogens rank second to *M. kansasii* as a cause of overt opportunist infection, but surveys of skin sensitivity suggest that subclinical infections may be relatively common and responsible for many of the non-specific reactions met in tuberculin tests. Some dysgonic nonchromogens have been identified as *M. avium* by pathogenicity tests on fowls (Marks & Birn, 1963), but further experience has shown that the fowl test is not completely specific. Lack of suitable means to classify members of the group, which is well recognized to comprise a number of different entities, has hindered the study of their epidemiology. The present investigation has applied to the problem of their classification a combination of serotyping by agglutination with specific antisera (Schaefer, 1965), lipid analysis by the thin-layer chromatography of extracts (Marks & Szulga, 1965) and certain cultural and biochemical examinations chosen for their utility in subdividing the group. The majority of the strains were also tested for virulence in fowls, although the results were not considered decisive for the present classification. The material examined consisted of the dysgonic nonchromogens isolated from man in Wales since 1953 together with strains sent to the Reference Laboratory for identification from England since 1959.

METHODS

Strains were stored at 4° C., and were subcultured every 3 months on Löwenstein–Jensen medium at 37° C.

Serotyping

Subcultures were made in Dubos medium, plated when grown on oleic acid-albumin agar plates and examined for agglutinability by the methods described

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previously (Schaefer, 1965). Most of the strains were satisfactorily typed, but a number either failed to disperse adequately or agglutinated spontaneously during the test. With some of these a later subculture from egg proved satisfactory. Others yielded satisfactory suspensions but were not agglutinated by any of the absorbed sera then available; such strains have been designated 'unclassified'. Four strains agglutinated with two different sera, and with one strain two types of colony reacting differently were found. One of those reacting with two different sera appears in Table 2, but otherwise the serotype consistent with the remaining properties has been used in classification.

Lipid analysis

The dysgonic nature of the organisms studied made a change necessary in the method used previously to prepare extracts. Volumes of 10 ml. of Löwenstein–Jensen medium were inspissated in 120 ml. medical flat screw-capped bottles kept at an angle of 45° after which 15 ml. of Kirchner's medium, modified by the use of Tryptone (Oxoid) instead of asparagine and 0·1 % bovine serum albumin instead of 10 % serum, were added to each. Care was taken in dispensing to minimize dispersal of the egg medium over the walls of the bottle and subsequently to avoid detaching any of the solid into the liquid phase. The latter layer was inoculated and the bottles incubated vertically at 37° C until the cultures were well grown—usually 4 weeks. The bacilli were harvested by centrifuging the liquid layer after its removal by pipette. The deposits were transferred to tared sample tubes, dried over P_2O_5 in a partial vacuum of about 60 cm. Hg for 48 hr. at room temperature and their 'dry' weight obtained. They were then extracted overnight (it was found later that 24 hr. is preferable) at room temperature with a solvent made of ethyl ether 43 volumes, ethanol 43 and water 14, the tubes being closed with silicone rubber bungs. The solvent was used in the proportion of 16 μl. for each mg. dry weight of bacteria. If the bacterial mass fell short of 15 mg., it was made up to this weight with water and 0·24 ml. of solvent used; however, the rare sample below 10 mg. was discarded in favour of a fresh culture. The chromatography of the extracts on thin-layer silica gel and the subsequent spraying with orcinol-sulphuric acid followed the methods described by Marks & Szulga (1965). As in their case, solvent 1 for two-dimensional analysis was propanol-ammonia run for 10 cm. and solvent 2 was butanol–acetic acid–water run for 7·5 cm. Each was used also for uni-dimensional runs as was solvent 3, propanol–isopropyl ether–water run for 10 cm.

Cultural and biochemical methods

Morphology was examined in Ziehl–Neelsen stained films of cultures on egg medium of the Löwenstein–Jensen type, in most cases using the primary growth. To examine the rate of growth at different temperatures, a loopful of growth from egg was ground with a drop of sterile water and 1 mm. loopfuls of suspension subcultured on four 2 ml. slopes of egg medium in 7 ml. screw-capped bottles which were then incubated at 25, 37, 42 and 45° C. respectively. Growth is often very fine and readings were therefore made with a × 5 hand lens. Dubious growth

was recorded as negative. Similar slopes were inoculated directly from the primary growth for incubation at 37° C., one in continuous light, the other in darkness. When the growth on these was mature their pigmentation was inspected.

Arylsulphatase was demonstrated by the method described by Marks & Trollope (1960) except that the substrate concentration was 0·001 M. The normal period of incubation used was 2 weeks, but a further week was allowed when growth was poor. The colour due to released phenolphthalein obtained by adding alkali was arbitrarily graded as +, ± or negative. Barely detectable tints were classed as negative.

The 2 ml. slopes of egg medium described above were also used for sensitivity tests, drugs being dispensed in them with twofold differences in concentration. A single minimal heating was used to solidify the medium. The test inoculum was a 2 mm. loopful (withdrawn edgewise) of a suspension prepared by emulsifying a loopful of growth from egg medium in 2 ml. of sterile water. The tests were read after 3 weeks' incubation. When severe but incomplete inhibition occurred in the last tube showing growth, an intermediate value was taken for the minimal inhibitory concentration (M.I.C.). Growth was assessed on the number of colonies and not their size.

Pathogenicity

The procedure for tests on fowls was finally standardized as the intravenous inoculation of 0·01 mg. moist weight of bacilli grown in Dubos medium. The fowls were previously shown to be negative reactors to 0·05 mg. of avian P.P.D. injected into the wattle; in most cases birds 6–10 weeks old were used. Before this method was evolved, however, a minority of the strains tested were inoculated either by a combination of the intramuscular and intraperitoneal routes (0·25 mg. by each) or similarly with the addition of 0·002 mg. bacilli intravenously. Wet weight was estimated by measuring the volume of centrifuged bacilli in a vaccine tube and taking 1 μl. as equivalent to 1 mg. or by matching the opacity of a Dubos culture with another estimated in the former manner. With 20 strains, the fowl test was supplemented by the intravenous inoculation of a rabbit with 0·01 mg bacilli. No strain was pathogenic for a rabbit which was harmless to a fowl, and some strains lethal to fowls produced only limited disease in rabbits. The rabbit test was not found helpful therefore and will not be considered further. The liver and spleen of fowls which died or were killed after 8–12 weeks were examined histologically and by culture. Lung and kidney were less useful and their examination was omitted in many cases.

Following the work of Scammon, Froman & Will (1964), a similar attempt was made to enhance the virulence of a number of strains, previously shown non-pathogenic for fowls, by growth at 42° C. Five strains were subcultured six times at weekly intervals in Dubos medium at 37° and 42° C. in parallel. Each of the final cultures was inoculated intravenously into three fowls using doses of 0·01, 0·1 and 1 mg. respectively, giving a total of 30 birds. Examination was as described above, survivors being killed after 12 weeks.

Table 1. *Properties of mycobacteria classified as* M. avium *and 'para-avian bacilli' isolated from man in England and Wales.*

(The number of strains is given for each property and the normal serotype and lipid pattern given in parentheses for each type.)

Proposed classification	Total no. strains	Normal serotype (Schaefer)	Normal lipid pattern	Virulence for fowl*			Sensitive to†		Arylsulphatase‡			Growth on egg at		Strains clinically significant
				+	±	–	Cycloserine	Ethionamide	+	±	–	42° C	45° C	
M. avium														
Type 1	13	8 (I)§	12 (A_1)	0	2	8	13	0	0	2	11	13	10	8
Type 2	24	21 (II)§	21 (A_2)	22	1	1	24	0	0	1	23	24	2	22
Para-avian bacilli														
Type 1	3	3 (IV)	3 (A_3)	0	1	1	3	3	2	1	0	3	3	1
Type 2	3	3 (VI)	3 (A_3)	1	0	1	3	3	0	1	2	3	3	3

* Five strains were not tested in fowls.

† Strains were defined as sensitive when the M.I.C. of cycloserine or ethionamide was 40 μg./ml. or less.

‡ Arylsulphatase activity is recorded as + when a deep red colour was obtained in the test and as ± with a pink colour.

§ Of the non-conforming strains, three had relatively non-specific serotypes and the rest were rough and could not be typed.

RESULTS

The present study is based on a series of 68 strains of dysgonic nonchromogenic opportunist mycobacteria isolated from different patients in England and Wales. In 54 cases the strain was defined as 'significant' on the criteria of repeated isolation or of culture from a biopsy or pus specimen, often supported by other evidence, whilst in 14 cases the strain was considered 'casual' or information was lacking. When all the properties studied were considered together, most of the strains could be assembled into four groups, of which three appear to merit recognition as species,

Table 2. *Properties of dysgonic nonchromogenic mycobacteria isolated from man in England and Wales and included in two provisional new species*

Group and strain no.	Serotype (Schaefer)	Lipid pattern	Virulence for fowl	Sensitive to*		Arylsulphatase†	Growth on egg at		Clinical significance
				Cycloserine	Ethionamide		42° C	45° C	
Provisional species 1									
18730	Boone	B	–	–	+	+	+	+	–
23774	VI	B	N.T.	–	+	±	+	+	+
24657	II + VI	B	–	–	+	+	+	+	+
25356	Rough	B	–	–	+	+	+	+	+
Provisonal species 2									
10792	Unclassified	C	N.T.	+	+	–	–	–	+
18698	Unclassified	C	N.T.	+	+	±	–	–	+
27479	Unclassified	C	N.T.	+	+	±	–	–	+
31042	Unclassified	C	N.T.	+	+	–	–	–	–
31242	Boone	C	N.T.	+	+	–	–	–	+

N.T. = Not tested.

* Strains were defined as sensitive (+) when the M.I.C. of cycloserine or ethionamide was $\leqslant$ 40 μg./ml.

† Arylsulphatase activity is recorded as + when a deep red colour was obtained in the test and as ± with a pink colour.

but 16 strains remain which are no doubt representatives of rarer species. Over half the strains have been assigned to the species *Mycobacterium avium* for which a new description is proposed below. Within this species, two types have been recognized in the present material, the most important properties of which are presented in Table 1. In the same table appear the properties of a group of six strains which appear to be closely related to *M. avium* and have therefore been called 'para-avian bacilli'. They are also divisible into two types. The two further groups have for convenience been called 'Provisional species 1 and 2'. Their chief properties are presented in Table 2 and those of the remaining, unclassified strains in Table 3. The results of each type of examination follow but formal descriptions of the groups proposed for recognition as entities will be deferred until the Discussion.

Morphology and cultural properties

All strains were acid- and alcohol-fast and nearly all exhibited short bacilli in films of growth on egg medium. However, seven *M. avium*, type 1 strains and two of Provisional species 1 presented long bacilli. The term 'dysgonic' applied to the

organisms discussed is relative and refers to their effuse and rather slow growth on egg medium. This type of growth appears to be associated with microaerophilism in mycobacteria; all the strains tested were weak in catalase activity and grew deep in semi-solid medium. With the pigmentation test described above, all members of the series were buff-coloured except one. This was a type 2 para-avian bacillus considered nonchromogenic initially but later found to produce a weak and inconstant yellow pigment.

Table 3. *Properties of dysgonic nonchromogenic mycobacteria isolated from man in England and Wales and not yet assembled in groups*

				Sensitive* to			Growth on egg at		
Strain no.	Serotype (Schaefer)	Distinctive lipid pattern	Virulence for fowl	Cycloserine	Ethionamide	Arylsulphatase*	42° C	45° C	Clinical significance
3897	Boone	+	–	+	–	±	+	–	+
18587	Boone	–	N.T.*	+	–	–	+	–	+
35445	Davis	+	+†	+	–	±	+	+	+
21233	Lunning	+	±	–	–	±	+	–	+
25633	Watson	+	–	+	+	–	+	+	+
653	Yandle	+	–	+	–	–	+	–	+
14546	I	+	–	–	+	–	+	–	+
2142	I	–	N.T.	–	+	–	±	–	–
7468	III	+	N.T.	+	–	–	–	–	+
326	Unclassified	+	N.T.	+	+	±	–	–	–
13444	Unclassified	+	±	–	+	+	+	±	+
18332	Unclassified	+	N.T.	–	–	–	+	+	–
35336	Unclassified	+	N.T.	–	–	–	+	–	+
11961	Rough	–	–	+	–	±	+	±	+
29494	Rough	–	N.T.	+	+	–	±	–	+
37176	Rough	–	–	–	+	–	+	–	+

Non-distinctive lipid patterns presented only features common to most mycobacteria; the 'distinctive' patterns differed amongst themselves.

* See Table 2.

† Virulence was attenuated after 4 years' storage.

About two-thirds of the strains in the series were grown in semi-solid medium as described by Marks & Richards (1962). All proved to be microaerophilic on the criterion of growth 10 mm. or more deep. However, when incubation was continued for more than a week, some strains grew more densely near the surface.

All members of the series grew on egg medium at 25° and 37° C. and, except for the five strains of Provisional species 2 and two unclassified strains, also at 42° C. Growth at 45° C. was a feature of Provisional species 1, usual with strains of *M. avium*, type 1 but uncommon with *M. avium*, type 2.

With rare exceptions, growth from egg medium emulsified easily, the bacilli in thin films being well dispersed. Similarly, growth in liquid medium was almost always diffuse.

Biochemical properties

It has been found useful in classification to distinguish between strains which have an M.I.C. with sulphonamide*, cycloserine and ethionamide $\leqslant$ 40 μg./ml. in Löwenstein–Jensen medium, here called *sensitive*, and those with an M.I.C. > 40 μg./ml., here called *resistant*. All the strains which have been classed as *M. avium* were sensitive to cycloserine and resistant to ethionamide on these criteria. Those classed as para-avian bacilli were sensitive to both cycloserine and ethionamide although the M.I.C. of ethionamide was fairly high and barely escaped a category of resistance. Strains placed in Provisional species 1 were resistant to cycloserine and sensitive to ethionamide and those in Provisional species 2 were sensitive to both drugs. Unclassified strains varied in their sensitivity pattern.

All the strains in Provisional species 1 and one thought to be related were sensitive to sulphonamide. Members of type 2 *M. avium* were almost all sensitive or gave borderline results. Otherwise only three strains were sensitive, one para-avian and two unclassified.

Arylsulphatase activity was absent in strains classified as *M. avium* except for three which were weakly positive. Two para-avian, type 1 strains were strongly positive and it is of interest that neither was clinically significant. Members of Provisional species 1 were arylsulphatase positive; those of species 2 were negative or weakly positive. Of the unclassified strains, one was strongly positive, five weakly positive and ten negative.

Details of the biochemical findings are presented in Tables 1, 2 and 3 except for sulphonamide sensitivity in which the M.I.C. is sometimes not clearly defined.

Serotyping

A total of 32 strains belonged to serotypes I and II which are held by Schaefer (1965) to be characteristic of organisms responsible for natural tuberculosis in birds. Of these, 29 are accommodated in the species *M. avium* as presently defined whilst one which gave a mixed serotype II and VI reaction is assigned to provisional species 1 and two of serotype I remain unclassified. The third most common serotype was VI which besides the mixed reaction noted above was found in six strains. These were distributed between the *M. avium*, para-avian and Provisional species 1 groups and the antigen therefore appears to make only a limited contribution to classification. This conclusion is supported by our finding antigen VI in two of three strains of *M. ulcerans* examined in a separate study. Serotype III was only met twice, in one case as a mixture with serotype II which was preferred for classification because of its link with the strain's lipid structure. Three strains assigned to the para-avian group (type 1) were serotype IV.

The remaining strains with a recognized serotype comprised five with Boone antigen and one each with Davis, Lunning, Watson and Yandle antigens ('Battey' serotypes—Runyon, 1959; Schaefer, 1965). One Boone strain was classified as *M. avium*, type 2 on its other properties and one each as Provisional species 1 and 2.

* 4-Sulphanilamido-5,6-dimethoxypyrimidine (Fanasil-Roche).

Two Boone strains and the other four named could not be assigned to any group. The Boone antigen thus appears to be even less useful in classification than VI.

Eight strains provided smooth suspensions but were not agglutinated by the set of sera then available; these appear in Tables 2 and 3 as 'unclassified'. Nine strains could not be tested because of rough suspensions and although in repeat attempts two of these gave acceptable suspensions, neither could be typed; all nine will be listed as 'rough'.

It will be noted in Table 1 that eight strains of *M. avium* lacked the characteristic serotype. The anomalies were as follows:

M. avium, type 1 (normal serotype I). Four strains were rough, one serotype VI.

M. avium, type 2 (normal serotype II). One strain was rough, one serotype VI and one Boone.

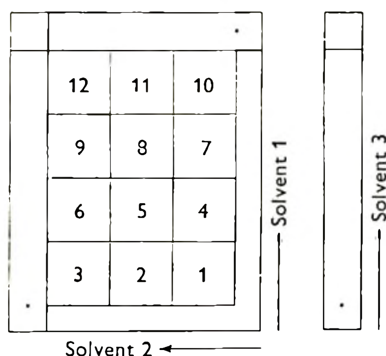


Fig. 1

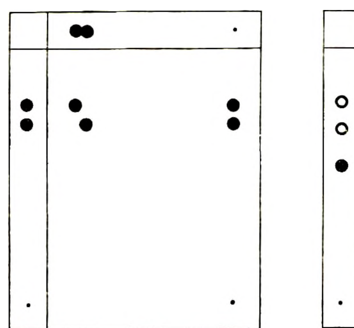


Fig. 2

Fig. 1. Diagram for reference in the text to positions of lipid spots. The numbered areas represent 2·5 cm. squares. The use of the solvents is described in the section on Methods. Samples for analysis were applied at the sites indicated by dots.

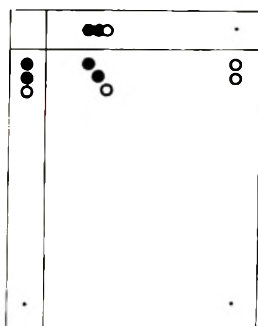
Fig. 2. Unidimensional and two-dimensional chromatographs of the characteristic lipids presented by strains of *M. avium*, type 1. The solvents and their direction of flow are as shown in Fig. 1.

Lipid structure

For ease of reference a diagram is given in Fig. 1 of a two-dimensional chromatogram divided into numbered 2·5 cm. squares. The directions are indicated of the flow of solvent 1 (propanol-ammonia) which was run for 10 cm. and of solvent 2 (butanol–acetic acid–water), run for 7·5 cm. All results refer to thin-layer chromatography on silica gel read after a spray with orcinol–sulphuric acid followed by heating. For clarity, non-specific lipids and substances derived from the medium have not been represented in the figures. With the two-dimensional runs the former almost all occur in squares 1, 2, 4 and 5 and the latter, which are faint, occur particularly at the lower margins of squares 7 and 8.

In Fig. 2 are presented the lipid patterns characteristic of extracts of *M. avium*, type 1, the unidimensional and two-dimensional results with solvents 1 and 2 appearing together, whilst alongside, the unidimensional 10 cm. run with solvent 3 (propanol–propyl ether–water) is depicted. This combination will be designated lipid pattern A_1. Its characteristic feature is the pair of spots which in the two-

dimensional run approximately straddle the lower margin of square 12 and always bear the same relative position to one another. In unidimensional runs in solvent 1 these spots are usually golden-brown but their colour in the two-dimensional run is often bluish grey. The difference appears to be due to a fall below a critical lipid concentration owing to splitting of the spots in the latter run, parts remaining immobile in solvent 2 as shown. The same effect on colour may be produced by over-spraying. Spots of moderate or high density are represented in the figures as solid circles, weak or inconstant spots as outlines; colour is not indicated. With solvent 3 the feature of pattern A_1 is a set of three rather weak brownish spots with R_F values of 0·55, 0·7 and 0·8 approximately. Unhelpful spots are again omitted from the illustration.



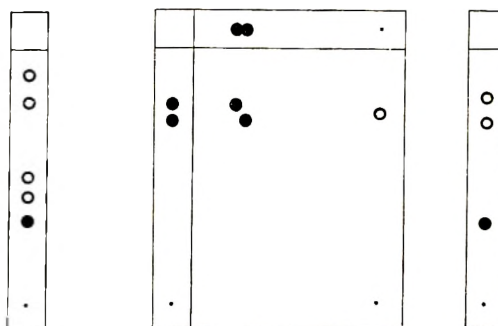


Fig. 3 Fig. 4

Fig. 3. Chromatrographs in the style of Fig. 2 of the lipids characteristic of *M. avium*, type 2.

Fig. 4. Chromatographs in the style of Fig. 2 of the lipids characteristic of mycobacteria classed as 'Para-avian bacilli'.

The lipid pattern characteristic of *M. avium*, type 2 and designated A_2 is shown in Fig. 3. In the two-dimensional run it presents a triad of spots on or adjoining the right-hand margin of square 12. With a non-specific elongated pink spot above which is not shown they form an arc which often continues to the upper left corner of the square. The lowest of the three spots shown is the weakest and is occasionally missing, but the upper two are stronger than the pair in pattern A_1 and almost always golden-brown besides occupying a different position. The middle spot is always much the densest of the three. One or two spots are usual on the right-hand margin of square 10 but they are weaker and less constant than those seen in pattern A_1. With solvent 3 there is a characteristic dense golden-brown spot at R_F 0·33 and usually two weak spots at R_F 0·43 and 0·5. These spots appear to correspond to the triad in square 12. Two faint brown spots also occur at R_F 0·8 and 0·9.

The para-avian bacilli of both types provided a lipid pattern A_3, which is presented in Fig. 4. With solvent 1 and 2 this pattern resembles A_1 although the R_F of the two main spots in solvent 2 is about 0·1 less, so that they are nearer to the lower right angle of square 12. With solvent 3 there is a dense brown spot of R_F 0·3

and two weaker spots of R_F 0·7 and 0·8 so that here the resemblance is more to pattern A_2.

Although the question did not arise in the main study, it is convenient to mention here that when three examples of SmT–SmD variation in *M. avium* were examined (Moehring & Solotorovsky, 1965), the SmD variants all exhibited the same lipid patterns as their parent strains. The cultures were kindly provided by Professor S. R. Pattyn, two strains being classified by us as *M. avium*, type 1, the other as *M. avium*, type 2.

When extracts of different strains in Provisional species 1 were examined in parallel, their similarity was obvious. Unfortunately, minor variations in technique appeared to affect the picture considerably, apparently owing to the low concentration of the lipids of interest. Because of this factor, unidimensional patterns

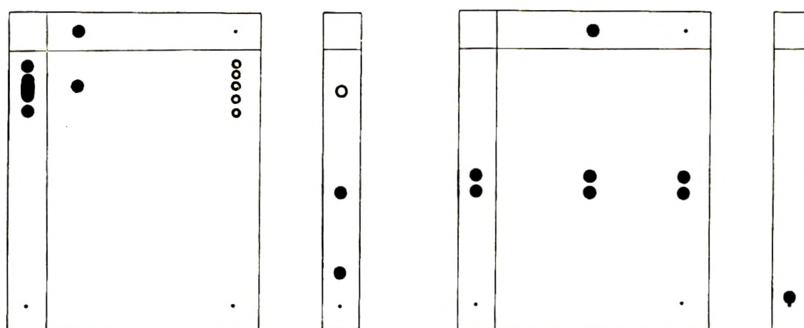


Fig. 5 Fig. 6

Fig. 5. Chromatographs in the style of Fig. 2 of the lipids characteristic of mycobacteria classed as 'Provisional species 1'.

Fig. 6. Chromatographs in the style of Fig. 2 of the lipids characteristic of mycobacteria classed as 'Provisional species 2'

were more consistent than two-dimensional. In Fig. 5, the pattern provided by the best extracts is presented. It has been designated B in Table 2. There are five weak bluish spots on the right margin of square 10 and a single spot near the centre of square 12. In the unidimensional runs some of these six spots fuse. With solvent 3 there is a weak bluish spot of R_F 0·13 and two weak brownish spots of R_F 0·45 and 0·85.

The lipid pattern characteristic of Provisional species 2 is presented in Fig. 6. It consists of a pair of spots having R_F values of 0·5 and 0·45 in solvent 1. Each splits into a stationary and a mobile component (R_F 0·5) in solvent 2. The spots have a yellow or greenish tinge in unidimensional runs but this colour is less distinct with runs in two dimensions. In solvent 3, a single spot is produced of R_F 0·05. With two strains (10792 and 18698), migration rates were 0·05 to 0·1 R_F greater in all three solvents and the faster of the two spots in solvent 1 was considerably denser than its fellow.

Amongst the ungrouped strains in Table 3, those with serotypes Watson, Davis and Lunning presented a rather similar pattern on lipid chromatography. Its chief

feature was a very prominent golden-brown spot at or near the lower right angle of square 12. In solvent 3, the R_F of this spot was 0·05, 0·1 and 0·2 respectively.

Virulence tests

The fowl tests recorded in the tables were carried out over a period of some years during which the dose and route of inoculation varied. The procedure which evolved from this experience was the intravenous inoculation of 0·01 mg. (moist weight) of bacilli grown in Dubos medium. This method was used for 30 strains. In 21 earlier tests a dose was used of 0·25 mg. of bacilli from Dubos medium intraperitoneally and 0·25 mg. intramuscularly. This was supplemented in nine cases by 0·002 mg. intravenously. In only six of the negative fowl tests had an intravenous dose been omitted, the strains being four of *M. avium*, type 1, one of Provisional species 1 (no. 24657) and one unclassified (no. 11961).

Six strains were tested for enhancement of virulence after their culture from the spleen of fowls which in five cases were judged negative histologically in the initial test and in the sixth case as showing limited lesions indicative of attenuated virulence. Each was re-inoculated intravenously into two fowls in a dose of 0·01 and 0·1 mg. respectively. Three strains were classified as *M. avium*, type 1, two as para-avian bacilli, type 1 and one as Provisional species 1 (serotype Boone). No increase of virulence was observed.

Five strains which showed no pathogenicity for fowls in their initial tests were retested after six parallel subcultures in Dubos medium at 37° and 42° C. using an intravenous dose of 0·01, 0·1 and 1 mg. for each of three fowls for each of the ten final cultures. The strains used for the experiment were one of *M. avium*, type 1, the only avirulent member of *M. avium*, type 2, one of para-avian bacilli, type 1, one of Provisional species 1 (serotype Boone) and one unclassified strain (serotype Watson). A certain degree of enhancement of virulence by growth at 42° C. as compared with growth at 37° C. was seen with the last two of the strains listed but only at the dose level of 1 mg. The pathogenicity thus elicited was of an order far below that of typical strains of *M. avium*, type 2 which are lethal with 0·01 mg. intravenously. Nevertheless, the results agree with those reported by Scammon *et al.* (1964).

In Table 1, a strain of *M. avium*, type 1 is recorded for convenience as being partially virulent. The first isolate from the patient was in fact lethal for a fowl, although not as destructive as a normal type 2 strain, but another obtained after 5 years' continued excretion of the organism proved to be avirulent although unchanged in its cultural properties.

DISCUSSION

It is widely recognized that the opportunist mycobacteria classed as dysgonic nonchromogens include diverse organisms despite their superficial similarity. Pathogenicity for the fowl is commonly used to distinguish *M. avium* amongst them, but even this limited contribution to the problem can mislead. The fowl test fails to identify certain members of the species which appear to have lost their

virulence for the natural host owing to prolonged residence in man and it occasionally misdiagnoses as *M. avium* the most pathogenic of the remaining dysgonic nonchromogens, which can be shown to be clearly distinct on other grounds. Serotyping has so far proved to be a promising means of classification but except where support is obtained by association with other discriminating properties or epidemiological evidence, the validity of any single character in classification must always remain in doubt. In fact, our findings suggest that certain antigens are so widely distributed that their contribution is of limited value. Moreover, a number of strains do not provide suitable suspensions for serotyping and a few appear to possess a mixture of the recognized antigens. The chief object of the present investigation was to determine to what extent the techniques of serotyping and lipid analysis could support one another as a basis for classification of the dysgonic nonchromogens. In the event, even these two means supplemented by certain biochemical, cultural and pathogenicity tests did not prove wholly successful, as almost a quarter of the strains in the series examined still await classification. However, considerable progress has been made which is embodied in the descriptions which follow of the entities recognized in our material. In the course of the work it became clear that the specificity of fowl tests would be improved by standardization of route and dose and the intravenous inoculation of 0·01 mg. moist weight of bacilli grown in Dubos medium is now recommended. Nevertheless, on the basis of this study it would appear that the fowl test is no guide to the clinical significance of a strain and plays only a subsidiary role in its classification.

The preliminary definition of the subject-matter of the present investigation and descriptions of the groupings identified in it now follow.

The 'dysgonic nonchromogenic mycobacteria'

Organisms in this category give on egg medium an effuse growth which takes at least a week to mature. Its colour is normally buff, but certain strains on continued incubation with ample aeration show some yellow pigmentation. The bacilli grown on egg are strongly acid- and alcohol-fast and most often short; they usually disperse well in suspensions and films. Growth extends at least 10 mm. below the surface in semi-solid medium and catalase activity is weak or absent. All strains in the present series grew at 25° and 37° C., almost all at 42° C. and many at 45° C. The majority were tested against the common anti-tuberculous drugs and, in comparison with normal *M. tuberculosis*, all were highly resistant to PAS and isoniazid and almost all to streptomycin, viomycin, kanamycin and thiosemicarbazone. Sensitivity to cycloserine, ethionamide and sulphonamide varied and proved useful in classification.

In the following descriptions of entities recognized within the dysgonic nonchromogens, the general properties of the latter will not be reported again except to note exceptional behaviour.

Mycobacterium avium

All members are sensitive to cycloserine and resistant to ethionamide on the criteria given above and lack arylsulphatase activity, with a few weakly positive

exceptions. The great majority of strains disclose recognizable lipid constitutions on the chromatography of extracts on silica gel. Two characteristic lipid patterns have been met which appear to be related since there is a general similarity and intermediate forms occur occasionally.

Most strains belong to Schaefer serotypes I or II. In the present series, a few strains were rough and unsuitable for serotyping and three, qualifying for the species on their other properties, belonged to the VI or Boone serotypes. The latter two antigens are widely distributed, however, and appear to be relatively non-specific.

In the present series of 68 dysgonic nonchromogens, 43 could be selected as possible members of *M. avium* simply on cycloserine, ethionamide and arylsulphatase tests. Six of these were then excluded primarily for having neither an appropriate serotype nor lipid structure. None of the 43 had strong arylsulphatase activity but three of the 37 strains accepted in the species were weakly positive compared with three of the six excluded. Even weak activity thus weighs somewhat against admission. Our experience suggests that *M. avium*, especially type 1 strains, can lose virulence for birds on residence in man and from the latter source are sometimes more accurately identified by drug and arylsulphatase tests than by the inoculation of fowls. Additional aids such as serotyping and lipid analysis improve the advantage of *in vitro* methods.

M. avium can be divided into two types on the basis of serotype and lipid structure. The validity of these divisions is supported by the incidence of certain other properties. Both types are known to cause natural tuberculosis in birds.

M. avium, *type* 1

Members are normally of Schaefer serotype I and exhibit the lipid pattern designated A_1. Only one of ten strains of this type isolated from man was highly pathogenic for a fowl and even then the usual florid picture of tuberculosis was muted; moreover a later isolate from the same patient was avirulent although culturally similar to the first. Most members of the type grow at 45° C. and about half of them exhibit long bacilli when grown on egg, an unusual property amongst dysgonic nonchromogens. Members are resistant to sulphonamide.

M. avium, *type* 2

Members are normally of Schaefer serotype II and exhibit the lipid pattern A_2. They are almost always pathogenic for fowls. Growth is always obtained at 42° C. but strains isolated from man seldom grow at 45° C. Most members are sensitive to sulphonamide or give a borderline result.

Para-avian bacilli

This name has been given to a group of organisms which appear to be closely related to *M. avium* but are excluded from the species by their greater sensitivity to ethionamide and sometimes by strong arylsulphatase activity. They exhibit a lipid pattern designated A_3 on chromatography of extracts which although apparently related to patterns A_1 and A_2, is distinct from either. The strains met so far are

divisible into types 1 and 2, the former of Schaefer serotype IV and the latter of serotype VI. All three para-avian type 2 strains met were isolated from cases of cervical adenitis.

Provisional new species 1

Four strains in the series were resistant to cycloserine, sensitive to ethionamide and sulphonamide, and arylsulphatase positive; they grew at 45° C. and were nonpathogenic for fowls (one not tested, however). This combination of properties appears to be distinctive and although serotyping has not been helpful, lipid analysis supports the recognition of the group as an entity. Three strains gave identical lipid patterns and that of the fourth (23774), although aberrant, appeared to be related. One further strain (13444) conformed to Provisional species 1 except in respect of lipid pattern and a moderate virulence for fowls. It has been placed for the present in the unclassified group.

Provisional new species 2

Only seven strains in the series failed to grow at 42° C. and of these five appeared to be related. These were sensitive to both cycloserine and ethionamide and either arylsulphatase negative or weakly positive. Serotyping did not assist classification; four of the strains failed to react with any of the sera available. However, the patterns observed on lipid chromatography were distinctive and supported recognition of the group as an entity.

SUMMARY

A broad division of the opportunist mycobacteria has been defined under the name of 'dysgonic nonchromogens'. The classification was attempted of 68 strains isolated from man in England and Wales, of which 54 at least appeared to be clinically significant. The means used were chiefly drug sensitivity, arylsulphatase activity, specific agglutination, lipid analysis and pathogenicity tests on fowls. On the results, a new definition is proposed for the species *M. avium*, extending beyond the boundaries of pathogenicity for birds, and a scheme put forward for its division into two types. In addition, two provisional new species and a group of 'para-avian' bacilli have been recognized and defined. The remaining 16 strains included six with 'Battey' serotypes but otherwise could not be classified. The need for standardization of fowl tests has been noted together with their diminished importance in the field of classification with the emergence of new *in vitro* methods of examination.

We are indebted to Prof. S. R. Pattyn for providing strains showing the SmT–SmD variation and to Mrs P. Duddridge and Mr A. Paull for technical assistance.

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