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Spontaneous magnitude estimation of thermal discomfort during changes in the ambient temperature*

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SUMMARY

Thermal comfort sensations are often studied in isolation, with the subjects' attention specifically directed towards their evaluation, both by instructions and by the recurrent act of questioning. A closer approach to the field situation, in which room temperature is at most a background stimulus, is made possible by the method of spontaneous magnitude estimation of thermal sensation. Thirty-six male and 36 female 17-year-old subjects in standard cotton uniforms (0·7 clo) were exposed in groups of 4 in a climate chamber to patterns of changing air temperature typical of conditions in occupied classrooms. Temperatures remained within the range 20–29° C. and did not increase more rapidly than 4° C./hr. Each individual recorded his thermal sensation on a dial voting apparatus, registering changes spontaneously as a secondary task while performing mental work during three successive 50 min. periods, with 10 min. breaks between. It was thus possible to obtain a measure of the time course of thermal discomfort sensations, including the extent to which they distracted attention. Significant differences were found between the responses of males and females, males in general feeling hotter and reacting more rapidly to changes in temperature. Response distributions are given in detail.

INTRODUCTION

Questionnaire methods currently in use to obtain measures of thermal comfort tend to decrease the inherent sensitivity of this sensation by using arbitrary category scales. Their reliance on what might be termed 'fixed-interval intrusion' and 'time-integrated retrospection' mean that it is difficult to study how such elusive time-variant factors as attention, arousal and effort may be affecting thermal comfort (Wyon, 1970*a*). Changes in these intervening variables can radically affect the relevance of thermal comfort in a real situation, as when a

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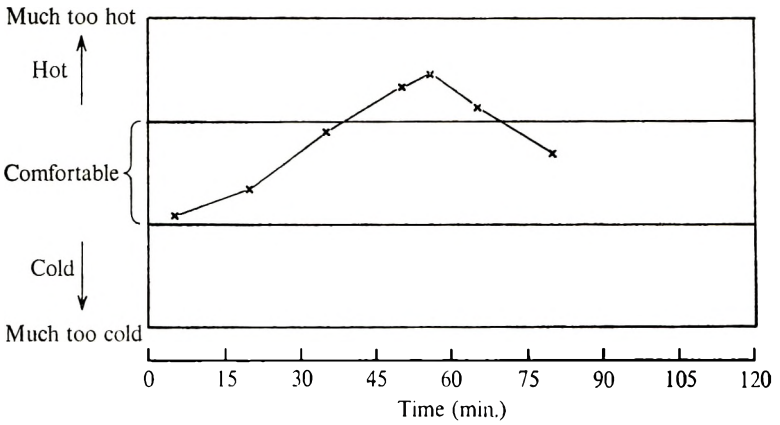

Fig. 1. Chart voting. Each subject has a chart on which he must place a cross whenever he becomes aware of his thermal sensation.

narrowing of the attentional field due to increased arousal or to concentration on performance leads to a marked reduction in the subjective importance of thermal discomfort stimuli. In such cases the intrusive act of questioning about thermal comfort can totally alter the attentional situation. This calls in question the validity of applying conventional questionnaire methods to the study of changes in thermal stress.

It is the purpose of this paper to propose a simple standard method for obtaining measures of thermal comfort and discomfort, a method which has some unique advantages for the study of subjective response to a changing thermal environment. The method is illustrated by its application in a study of such temperature changes as might occur in school classrooms, carried out in a climate chamber with 17-year-old pupils as subjects.

PILOT EXPERIMENT

Chart voting

As part of the development of the method, a preliminary experiment was carried out at the University of Aston in Birmingham (Wyon, 1968, unpublished).

Groups of about 25 17- to 19-year-old British students attending normal mathematics lectures in a lecture theatre with controlled-temperature air supply recorded their thermal discomfort sensation as a function of time on forms of the type shown in Fig. 1.

Their instructions were to place a cross on the chart whenever they became aware of their thermal discomfort sensation in the course of the lecture, taking care to locate it correctly on the time axis (abscissa). The position of the cross on the thermal discomfort sensation axis (ordinate) should indicate their thermal vote at that time on a continuous scale from 'much too cold' to 'much too hot'. There were no objections from the subjects to the idea of a comfort zone occupying one-third full scale. In all, over 120 students took part, each group being exposed to a different rate of rise of temperature. Difficulties in controlling the temperature and temperature differences from front to back of the steeply ramped lecture

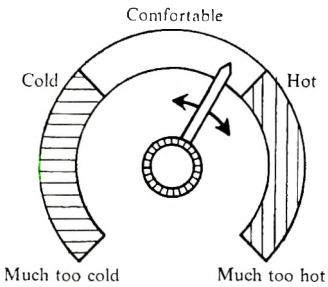

Fig. 2. The dial on the dial vote apparatus is divided in three zones: a cold, a comfortable and a hot zone. The pointer is moved to a new position, whenever the subject becomes aware that his thermal sensation has changed.

theatre made it difficult to discriminate effects of rate of rise *per se*, but it was clear that the subjects could record meaningful estimates of thermal discomfort sensation in this way, and that they responded to changes of less than 1° C. in air temperature.

When experiments must be carried out on large groups of subjects, it is justifiable on practical grounds to complicate their task by giving them the responsibility of recording time, but it does introduce a source of error as well as adding to their work load. Experiments on individuals or small groups can with advantage be carried out using the 'dial vote' version described in the following section.

METHOD

Dial voting

Each subject was provided with a dial as shown in Fig. 2. It resembled a thermostat control, with the range of adjustment covering a 270° angle, divided clearly into three equal zones, each 90°. The central zone was green, and marked 'comfortable', the zone to the left of centre was blue and marked 'cold', and the zone to the right of centre was red and marked 'hot'. A pointer could be set to remain at any point on the scale, whose extremes were marked 'much too cold' and 'much too hot' as shown. No other graduation or wording appeared on the scale. Subjects were instructed to ensure that the deviation of the pointer from the centre of the scale always represented the magnitude of their thermal discomfort sensation, and it was pointed out to them that this implied that they should alter the setting whenever they became aware that their thermal discomfort sensation had changed. It was emphasized that they would not be reminded to make these adjustments during the experiment, and must remember to do so themselves. They were not given any idea of whether the temperature would go up or down or remain constant; indeed, if they asked, it was emphasized that they did not know and should therefore record their sensation rather than their expectation. The dial faces were recessed so that each subject could only see his own dial setting. Subjects were told that the end-points of the scale represented unbearable extremes, where they would be obliged to leave the climate chamber, and that such extremes would not occur in the experiment. This instruction was intended to discourage subjects

Table 1. *Physical characteristics of subjects*

Group	No.	Age (years)	Height (cm.)	Weight (kg.)	Du Bois area ($m.^2$)	Skin-fold thickness (mm.)
Males	36	16·6 ± 0·9 (15–18)	179·0 ± 5·0 (169–191)	64·9 ± 7·0 (54–79)	1·81 ± 0·11 (1·63–2·00)	70·2 ± 18·7 (47–108)
Females	36	16·4 ± 0·7 (15–18)	162·2 ± 5·9 (151–180)	53·9 ± 4·9 (45–64)	1·56 ± 0·08 (1·42–1·81)	90·4 ± 18·8 (68–154)

Mean values ± 1 standard deviation. Ranges in parentheses. Skin-fold thickness is the sum of measurements at five sites for each subject: mid-axillary, supra-iliae, abdominal, triceps and thigh.

from making settings at or close to the end-points, thus avoiding compression of scale. The results suggest that this aim was achieved, as the scale was linearly related both to temperature and to the Bedford scale.

By connecting each pointer to a potentiometer in a simple bridge circuit the setting on each dial was remotely and continuously recorded in such a way that readings were available every 72 sec. with an accuracy of 1 % of full scale. It was ensured that settings in the three zones actually gave values of 0–33, 34–66, 67–100 respectively, so that transitions between comfort and discomfort were accurately recorded. The scale was linear with respect to dial setting.

Experiment

The subjects were 72 healthy Danish high-school pupils of high ability; they were volunteers, but paid by the hour; each subject attended only once. None of them were acclimatized to heat. The physical characteristics of the male and female groups, each of 36 subjects, are given in Table 1.

Denmark has a temperate Atlantic climate, and during the experimental period from 17 September to 10 October 1969 the local average maximum and minimum outdoor temperatures were 15·1° C. (range 10–21° C.) and 8·9° C. (range 2–14° C.) respectively. The experimental period was preceded by stable outdoor conditions at a slightly higher temperature level.

Three different temperature conditions were used in the climate chamber exposures. The control condition (condition 0 in Fig. 4) was a saw-tooth variation of temperature, rising from 20 to 23° C. in the course of 50 min., then decreasing to 20° C. in the rest period of 10 min. This temperature variation was repeated twice during the next 2 hr. In condition 1 (see Fig. 4) a base-line increase of 1° C. per hour was imposed on the saw-tooth variation of condition 0, with the result that the temperature rose 4° C. in 50 min. and fell 3° C. in the next 10 min., starting as for condition 0 at 20° C. and thus ending at 26° C. after 3 hr. In condition 2 the same variation occurred but at a higher level; the temperature was initially 23° C. and thus became 29° C. after 3 hr. (see Fig. 4).

The temperature was measured as the arithmetic mean of air temperature and radiation temperature and will in the following be referred to as temperature only. The chamber was especially designed for dynamic studies, and the walls inside the

insulation have a very low thermal capacity. The absolute humidity of the air was constant at 9 mm. Hg (corresponding to 50 % R.H. at 20° C.), the air velocity was 12 ± 3 cm./sec. and the air supply rate was 600 m.^{3}/hr.; there was no recirculation. Further data on the performance of the climate chamber are given by Andersen & Lundqvist (1970).

Procedure

The subjects attended school as usual during the first 3 hr. of the day; after lunch each day single-sex groups of four were taken by car to the climate laboratory, where they changed into standard cotton-drill suits (Plate 1). Cotton shorts and a cotton T-shirt were worn underneath, together with cotton socks and light canvas shoes. The total insulation value of this ensemble was 0·7 clo. As the suits are very similar to the denim jeans and jackets that are frequently worn in school by Danish pupils of this age, both male and female, they can be considered a fairly typical 'basic school clothing'. In practice, there will be more deviations towards higher insulation values, as when a woollen sweater is worn, than towards lower insulation. As the present experiment was intended to derive recommended maximum values for classroom temperatures, it would have been inappropriate to take account of the effect of extra items of clothing. It is both cheaper and more effective to insist that such extra items be removed than to ensure that classroom temperatures should be lowered on their account. On entering the climate chamber, subjects were seated at individual desks facing a fifth desk used by one of the experimenters (DPW), who was present throughout the total exposure period. The subjects worked continuously at a programme of psychological tests (the results of which will be presented in a forthcoming paper) and only in the rest periods were they allowed to relax and walk around in the chamber. Skin and rectal temperatures were recorded continuously and ECG records were taken at intervals. These and other physiological observations will be the subject of a further article.

The subjects were instructed in the use of the dial voting equipment as set out above. They were reminded at the start of each 50 min. period that they should adjust the setting spontaneously, but during each such period no mention of dial voting was made. At the beginning and end of each period, while still sitting at their desks in the climate chamber, the subjects also voted on the seven-category Bedford scale (Bedford, 1964).

RESULTS

Relation between Bedford scale and dial vote

Mean thermal votes obtained on the Bedford thermal voting scale at the beginning and end of each 50 min. period are shown in Fig. 3 for males and females separately. Conventional questionnaire voting was limited to these two occasions to interfere as little as possible with the spontaneous thermal votes. The temperature variations (Fig. 4, lower section) are clearly reflected in the variation of the mean thermal votes (Fig. 3), and also in that of the mean dial votes (Fig. 4). It was noticed that individuals often adjusted their dial vote setting by as little as 1 %

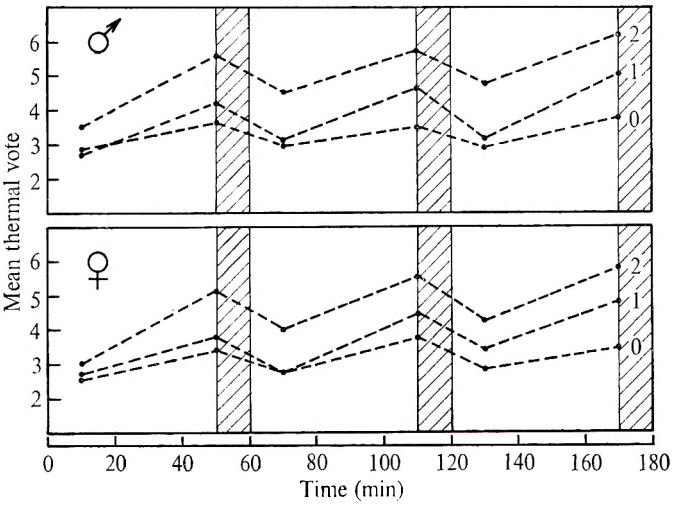

Fig. 3. The mean thermal votes on the seven-point Bedford scale obtained at the beginning and end of each hour of the three experimental conditions 0, 1 and 2. On this scale 4 is the centre (ideal comfort), 1 and 7 much too cold and much too hot respectively and 2 and 6 too cold and too hot respectively. The hatched columns represent rest periods with a 3° C. decrease in temperature.

full scale, i.e. they used the possibility of making much finer discriminations than are possible with the Bedford seven-category scale. A comparison has been made between the thermal votes recorded by each subject at the beginning and end of each period and the simultaneous dial vote setting. These scatter diagrams are shown in Fig. 5(*a*) and (*b*) for males and females respectively, and no systematic deviations from a straight line are apparent. The Pearson correlation coefficient for males is 0·85 and for females 0·84, and the corresponding linear regression equations are $D = 12{\cdot}4B + 4{\cdot}2$ and $D = 11{\cdot}9B + 2{\cdot}7$ respectively (where D is the dial vote and B the Bedford scale vote). The regression lines do not differ significantly in any respect. In addition, no significant differences were found between regression lines drawn separately for votes at the beginning and end of each session, for either sex.

Dial voting therefore appears to result in a measure of thermal comfort fully comparable with the Bedford scale. Since in addition it overcomes the general objections to the use of questionnaire methods that were raised in the introduction, it would appear well suited to the investigation of changing temperatures. Results obtained by means of dial voting are analysed in the following sections.

Analysis of variance of dial votes

Dial vote settings were evaluated at regular intervals of about 5 min. (4 timing units, equal to 4·8 min.) for each person separately throughout his or her exposure. They were expressed as a whole-number percentage of full scale, with 0 and 100 as the extremes of cold and hot discomfort respectively. Thus the comfort zone encompassed 34–66, and 50 represented ideal comfort. Fig. 4 shows how the mean

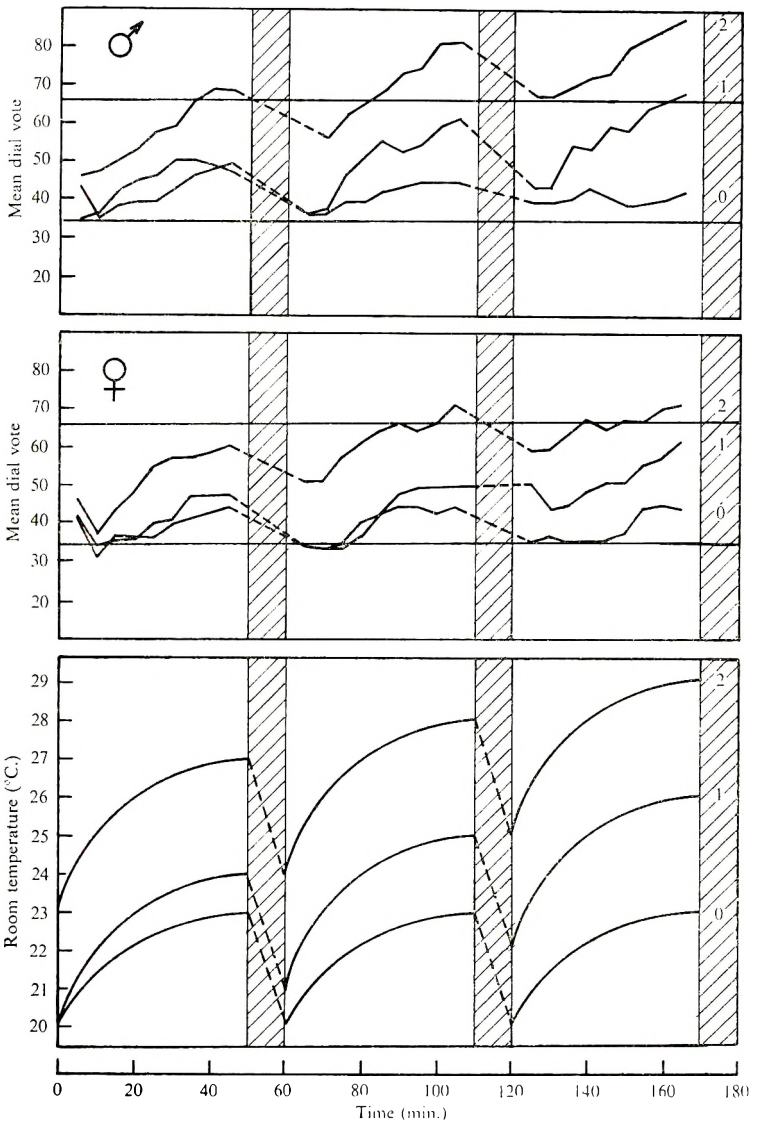

Fig. 4. The two upper sets of curves show the variation with time of the mean dial votes. The boundaries between the cold and the comfortable zone and between the comfortable and the hot zone are shown as horizontal lines at 33 and 67 respectively. The lower set of curves show the variation with time of ambient temperature (surface temperatures = air temperature) during the three experimental conditions 0, 1 and 2. The hatched columns represent the rest periods.

setting for groups of 12 boys or girls changed with time over their three 1 hr. exposure periods, for each of the three temperature conditions.

In order to examine the significance of such differences as are apparent in the figure, analysis of variance was carried out with dial vote setting as dependent variable, for each temperature condition separately. Time and temperature effects are confounded throughout, as must be the case in any investigation in which subjects are exposed to temperatures that change during rather than between

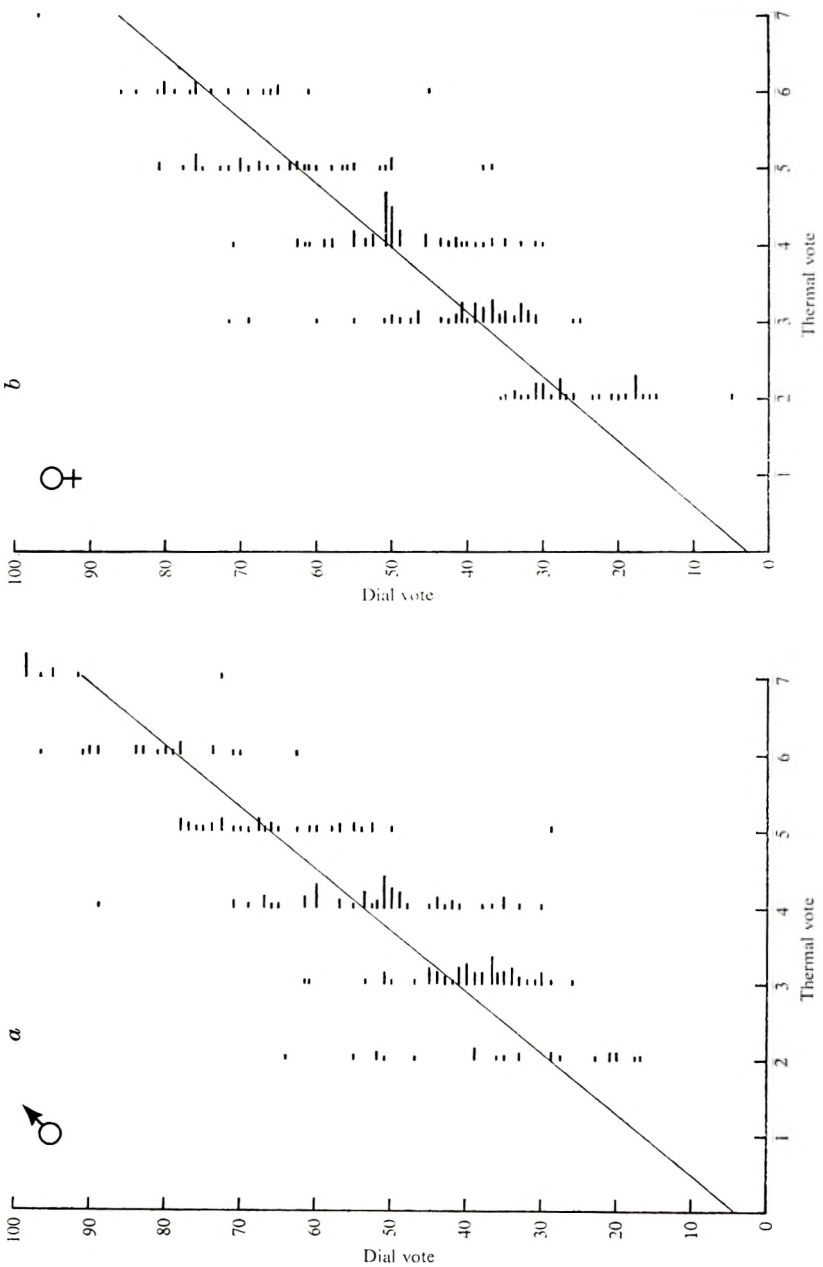

Fig. 5. The distribution of dial votes obtained simultaneously for each category of thermal vote for males and females separately. The shortest lines represent one subject, longer lines a proportionally greater number of subjects.

Table 2. *Dial vote: analysis of variance summary table for conditions* 0, 1, 2

(The analysis examines the effects of the three independent factors Sex, Periods and Minutes, taking dial vote as the dependent variable. Within a given temperature condition, each subject was present for three successive periods, each of 50 min. The analysis of variance takes account of the fact that the dependent variable was recorded repeatedly from the same subject in successive minutes and periods by deriving error terms that include the interaction, if any, between subjects and the relevant factor. (See Winer (1962), pp. 319–35.)

		Condition					
		0		1		2	
Source of variation	D.F.	MS	F	MS	F	MS	F
Between subjects							
Sex	1	751·86	< 1	5,910·26	4·49*	10,771·86	7·55*
Subjects within	22	1,543·86	—	1,317·59	—	1,426·43	—
Within subjects							
Periods	2	183·38	< 1	6,852·37	11·31***	14,450·04	26·46***
Sex × periods	2	38·45	< 1	337·86	< 1	420·15	< 1
Periods × subjects within groups	44	459·18	—	605·84	—	546·07	—
Minutes	8	720·33	9·74***	3,177·06	26·66***	3,803·83	44·42***
Sex × minutes	8	36·30	< 1	357·22	3·0**	193·94	2·26*
Minutes × subjects within groups	176	73·97	—	119·17	—	85·64	—
Periods × minutes	16	104·38	1·95*	103·16	1·25 NS	78·06	< 1
Sex × periods × minutes	16	69·26	1·30 NS	70·83	< 1	45·34	< 1
Periods × minutes × subjects within groups	352	53·42	—	82·28	—	86·47	—

NS Not significant. * Significant at 0·05 level. ** Significant at 0·01 level. *** Significant at 0·001 level.

successive exposures. As temperatures changed non-linearly with time it was decided to take time as the independent variable for the purpose of analysis. The factors investigated were Sex (2 levels), Periods (3 levels, for the three successive hours of exposure) and Minutes (9 levels, roughly 5 min. intervals, covering the first 40 min. of each exposure period). The final 10 min. of each period were disregarded, partly because Bedford scale voting took place towards the end, which could have affected dial vote settings, and partly because there was a certain amount of variation in the exact time at which chamber temperatures began to decrease at the end of each period. The statistical model for the analysis of variance was that of a three-factor experiment with repeated measures on the last two factors (Periods, Minutes). A full exposition of this model is given on pages 319–35 of Winer (1962). The total of 36 boys and 36 girls had been assigned at random to the three temperature conditions, so 12 boys and 12 girls experienced each condition.

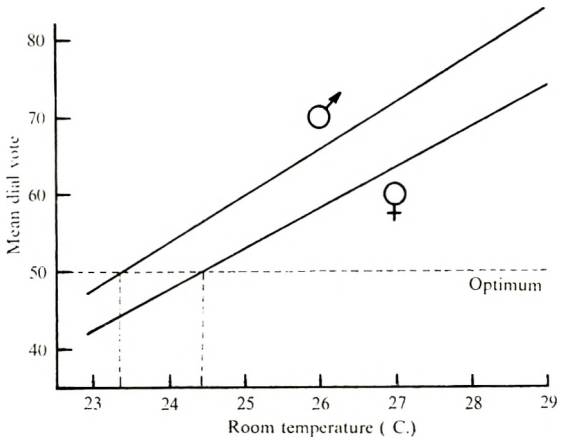

Fig. 6. Relation between temperature and mean dial vote for males and females separately.

Temperature condition 0 (20–23, 20–23, 20–23° C.)

Table 2 sets out the analysis of variance of dial votes in condition 0. There were no significant main effects of sex, and no interactions with the sex factor. There were no differences between periods, and here it should be remembered that there was in fact no base-line change in condition 0 – the temperature rose from 20 to 23° C. in all three periods. However, the main effect of time within each period ('Minutes') was significant ($P < 0{\cdot}01$), showing that subjects responded to the 3° C. temperature change even though most of the dial votes were within the comfort zone. There was a significant interaction between Periods and Minutes ($P < 0{\cdot}05$). An examination of Fig. 4 shows that dial votes tended to increase by a progressively smaller amount in the later periods.

Temperature conditions 1 (20–24, 21–25, 22–26° C.) *and* 2 (23–27, 24–28, 25–29° C.)

Table 2 sets out the analysis of variance of dial votes in conditions 1 and 2. The results are identical in each condition. The main effect of Sex was significant ($P < 0{\cdot}05$); boys felt significantly hotter than girls. The main effect of Periods was significant ($P < 0{\cdot}001$), and there was no interaction with Sex; boys and girls detected the base-line temperature rise of 1° C./hr. The main effect of Minutes was significant ($P < 0{\cdot}001$), and there was a significant interaction with Sex ($P < 0{\cdot}05$), though not with Periods. Subjects detected the temperature rise of 4° C. within each period, and the rate of increase of dial vote with temperature was the same in each period in spite of the base-line temperature rise. Boys tended to alter their dial vote settings by a greater amount than girls in response to a given temperature change.

Relation between dial vote and temperature

Correlation between mean dial vote and temperature

Values of the mean dial vote for each group of 4 subjects at 10, 30 and 50 min. from the start of each period have been used to calculate the regression lines shown

in Fig. 6. Boys and girls are shown separately. The scatter diagrams indicated a linear relationship in each case. It can be seen that girls tended to prefer a slightly higher temperature than boys, and there is a clear indication that girls increased their dial vote more slowly with temperature than boys. These points have been examined by means of analysis of variance and were shown to be significant. The correlation coefficients tended to increase towards the end of each hour, reaching 0·65 and 0·67 after 50 min. for boys and girls respectively. Optimum temperature for boys was 23·4° C. (dial vote 50) and the regression line remained in the comfort zone over a range of 5·5° C. (20·6–26·1° C.). Optimum temperature for girls was 24·4° C. and the regression line remained in the comfort zone over a range of 6·4° C. (21·2–27·6° C.). At the limits of these ranges, however, 50% of subjects would be uncomfortable, and a much narrower range of temperatures would be necessary to ensure that all subjects remained within their individual comfort zones. The following subsection deals with this point.

Comfort and discomfort distributions against temperature

By examination of the dial vote settings maintained by each individual at 10, 30 and 50 min. from the start of each hour, the proportions too cold, comfortable and too hot were obtained and related to the measured air temperature. Probit analysis was performed (Finney, 1947), yielding the calculated regression lines shown in Fig. 7. They show for each sex separately the expected proportions too hot and too cold at each temperature (left-hand probit scale). By subtraction, the proportion comfortable may be obtained; such distribution curves are shown in Fig. 7 for each sex separately (right-hand percentage scale). This presentation of comfort and discomfort distributions was suggested by Wyon, Lidwell & Williams (1968).

Where the probit regression lines intersect, the proportion too cold is equal to the proportion too hot. Any deviation from this point results in an overall increase in the total proportion uncomfortable. For boys, the lines intersect at 23·8° C.; for girls, at 25·0° C. However, for a mixed population the 'optimum' temperature is where the proportion of girls too cold is equal to the proportion of boys too hot. This point occurs in the present data at 24·3° C., at which temperature 16% of girls were too cold and 16% of boys were too hot. In determining the optimum temperature for any population it should be remembered that those too cold can always wear more clothing, whereas there is usually a limit to how much clothing those too hot can remove. The limiting factor in deciding classroom temperatures should perhaps be the expected proportion of boys who are still too hot when wearing a reasonable minimum of clothing. In the present experiment the clothing insulation (0·7 clo) was probably close to that of the usual clothing worn in schools by pupils of this age. On the other hand the activity level during the experiment was almost certainly lower than in normal school conditions owing to the lack of active recreation between periods, and the air velocity was somewhat higher than is common in school classrooms (Andersen & Lundqvist, 1966). Both of these factors will tend to reduce the temperatures required for comfort in practice. Where conditions do correspond to those of the experiment, a maximum tempera-

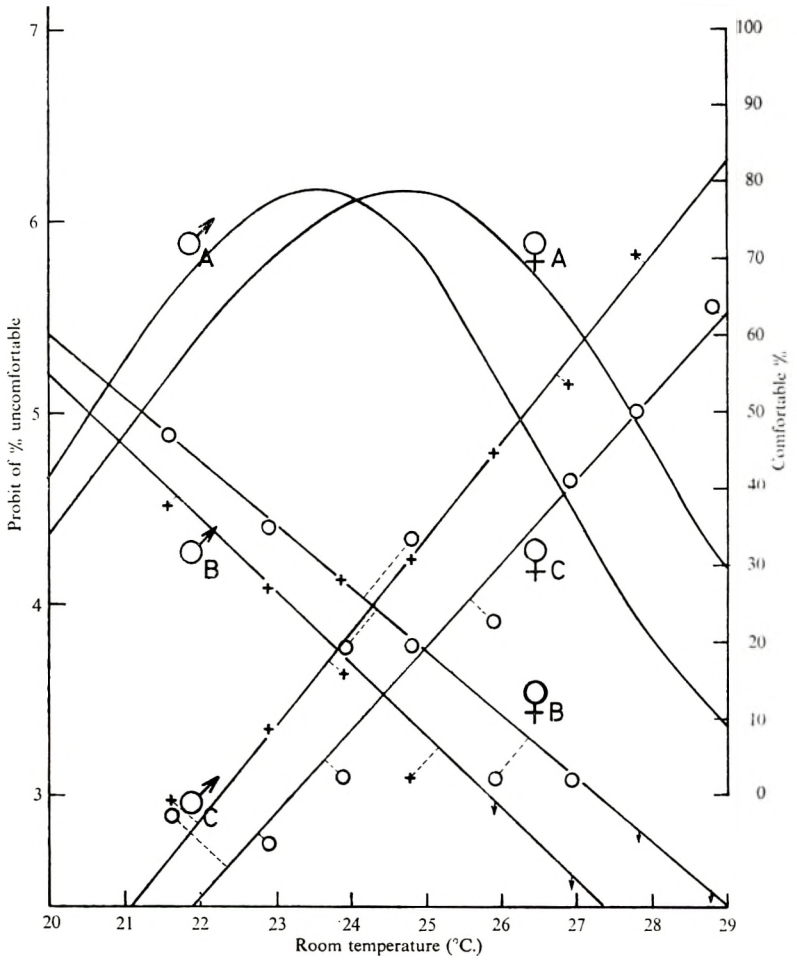

Fig. 7. Proportion of subjects comfortable, uncomfortably cold and uncomfortably hot at various temperatures. Males and females are shown separately. The two curves A show the percentage comfortable (right-hand scale). The two lines B show the probit of the percentage uncomfortably cold and the two lines C show similarly the uncomfortably hot (left-hand scale).

ture between 23 and 24° C. would seem reasonable (Fig. 7), for at 23·7° C. 10 % of boys were too hot (and 20 % of girls were too cold) and at 23·0° C. 5 % of boys were too hot (and 28 % of girls were too cold).

Comfort/discomfort dichotomy from dial vote-temporal effects

There is a quite explicit dichotomy between dial vote settings that are in the comfort zone and settings that are outside it. Using only this dichotomy, and thus avoiding any assumptions about scaling, it is possible to follow the time course of subjective response to imposed temperature changes. Two measures were derived to characterize a subject's response to the temperature change within a given 50 min. period: the number of minutes that elapsed before he first spontaneously made a dial vote setting in the 'too hot' zone, and the total number of minutes for which dial vote settings were maintained in the 'too hot' zone. The former

Table 3. *Mean number of minutes tolerated before vote of 'too hot' was recorded* (12 *subjects of each sex encountered each condition*)

	Boys			Girls		
Period ...	1	2	3	1	2	3
Temperature condition						
2	33·5	12·8	10·6	35·6	24·0	12·7
1	45·6	34·7	28·8	44·2	45·9	33·0
0	43·3	50·0	50·0	41·7	48·5	50·0

measure is applicable only to rising temperatures, while the latter is quite generally applicable to any pattern of rising and falling temperature. Both measures make use of the spontaneous nature of dial vote responses. Non-parametric statistics were used in the analysis (Siegel, 1956).

Table 3 shows the number of minutes for which dial vote settings remained within or below the comfort zone, averaged over 12 subjects of each sex, for each period of each temperature condition. It may be seen that (*a*) there was a tendency for subjects to become 'too hot' sooner in the warmer conditions, (*b*) boys tended to become 'too hot' sooner than girls, and (*c*) the base-line rise of 1° C. per hour during conditions 1 and 2 was reflected in the results. The significance of these effects was tested both for the criterion of 'minutes tolerated before vote of "too hot" recorded' and for the more general criterion of 'total minutes for which vote of "too hot" recorded', using the same procedures.

Kruskal-Wallis analysis was used to test the extent to which each criterion distinguished between temperature conditions. Values of ($P < 0{\cdot}001$) were obtained for each criterion, for boys and for girls separately. Clearly both sexes became 'too hot' sooner in the warmer conditions, and were uncomfortably hot for a greater proportion of each exposure.

The Mann–Whitney U-test was used to test differences between the responses obtained from each sex. No significant difference could be shown on the first criterion within any of the three conditions, but on the second criterion values of ($P < 0{\cdot}01$, $< 0{\cdot}05$, $\geqslant 0{\cdot}05$) were obtained within conditions 2, 1 and 0 respectively. Boys were 'too hot' for a significantly longer time than girls in the warmer conditions. Very few became 'too hot' in the control condition, rendering both criteria insensitive.

Friedman two-way analysis of variance was used to test whether subjects had detected the base-line rise between periods within each temperature condition. On the first criterion, a significant result ($P < 0{\cdot}01$) was obtained for condition 2 only, for each sex separately. On the second criterion, values of ($P < 0{\cdot}01$) were obtained for boys meeting condition 2 and condition 1, whereas a significant value ($P < 0{\cdot}05$) was obtained for girls only in condition 2. It should be remembered that there was no base-line rise between periods in the control condition 0. In condition 2 the base-line rise of 1° C./hr. caused both sexes to become 'too hot' sooner, and to remain 'too hot' for a longer time. In condition 1 the base-line rise caused boys to remain 'too hot' for a longer time.

Table 4. *Adjustments to dial vote in each condition* (12 boys + 12 *girls met each condition, total* 72 *subjects*)

Temp. condition ...		0	1	2
Total no. adjustments in 3 hr. (for each subject)	Boys	3, 3, 4, 6 6, 6, 7, 10 10, 11, 11, 17	2, 3, 7, 10 10, 13, 15, 19 20, 21, 23, 24	6, 8, 9, 10 10, 13, 15, 17 17, 17, 19, 24
	Girls	5, 6, 6, 8 10, 10, 11, 11 13, 17, 18, 18	5, 6, 6, 7 8, 8, 9, 9 9, 10, 10, 14	4, 5, 7, 8 11, 12, 15, 15 18, 18, 19, 20
Mean no. adjustments per person per hour	Boys	2·6	4·6	4·6
	Girls	3·7	2·8	4·2
Mean % time uncomfortable	Boys	21·7 %	33·5 %	59·8 %
	Girls	32·8 %	31·7 %	44·4 %

Discomfort as distraction

Subjects adjusted their dial vote whenever they became aware of their thermal sensation. The frequency with which they did so is thus a measure of the distracting nature of their sensations of thermal discomfort. Only if subjects vote spontaneously can this empirical information be obtained. Any form of elicited response, and particularly fixed-interval questioning, alters the attentional status of thermal sensations.

It seems reasonable that uncomfortable sensations should be more distracting than comfortable sensations. Alternatively, sensations which for some reason distract the subject's attention seem likely to be judged more uncomfortable than sensations which do not. In both cases it may be predicted that subjects voting in the uncomfortable region of the dial vote scale will be more aware of their thermal sensations and will therefore make more frequent adjustments to their dial vote settings. This prediction can be tested in two ways on the present material, as set out below.

Number of adjustments to dial vote in each hour

Table 4 sets out the total number of adjustments made by each subject in the course of his or her 3 hr. exposure, classified according to sex and the temperature condition encountered, and the mean number of adjustments per person per hour for each such group of 12 subjects. In order to decide empirically which temperature conditions were more uncomfortable, the proportion of the total exposure time for which each subject had maintained a dial vote setting of 'uncomfortable' (too hot or too cold) was calculated. Table 4 sets out the mean values obtained in this way for the groups of 12 subjects. It can be seen that for boys, condition 2 was more uncomfortable than condition 1, and condition 1 was more uncomfortable than condition 0. The prediction is therefore that boys would make the greatest number of adjustments to dial vote in condition 2, and the least number in

condition 0. For girls, condition 0 tended to be slightly too cold, so that condition 1 was the most comfortable and condition 2 the least comfortable. The prediction is therefore that girls could make the greatest number of adjustments to dial vote in condition 2, and the least number in condition 1. The data of Table 4 support these predictions. They may be tested on the individual adjustment data by means of the powerful distribution-free test against ordered alternatives due to Jonckheere (1954). This is a K-sample test of which the two-sample Mann–Whitney U-test used earlier is a special case. Without correction for ties, i.e. at a conservative estimate, the test yields ($P < 0{\cdot}05$) for each sex separately. Evidently subjects did tend to adjust their dial vote setting more frequently under the conditions that caused them to be uncomfortable for a greater proportion of the time.

Mean number of minutes for which each setting was maintained

If subjects adjust their dial vote setting more frequently, the time for which they maintain each setting must decrease on average. The prediction that the average time-lapse between setting will be less when settings are outside the comfort zone than when they are inside the comfort zone may be tested on the present data. If supported, it implies that subjects who are comfortable have longer periods in which they are not sufficiently distracted by their thermal sensation even to alter their dial vote setting. Forty-six of the 48 subjects who encountered condition 1 or condition 2 were both comfortable and uncomfortable at some time during their exposure. For each of these subjects a figure was derived for the length of time each setting had been maintained. Settings were divided into those in the comfort zone and those outside it, and for each subject the time-values linked to each category of settings were averaged and compared by means of the binomial test. One boy had exactly the same average time for settings inside and outside the comfort zone, but 18 out of the remaining 23 boys had longer average times for settings inside the comfort zone. This is significant at the ($P < 0{\cdot}005$) level for a 1-tail test. Thirteen out of 22 girls had longer average times for settings inside the comfort zone, and 9 the reverse. This result does not approach significance. The prediction is thus confirmed at the 0·005 level for boys, but is not confirmed for girls.

Since the pattern of rising temperature usually led to more votes of heat discomfort being recorded later in the exposure, a tendency to alter settings more frequently later in the exposure could have given rise to the above result. The existence of such a time effect was therefore tested on the control group of 24 subjects who encountered condition 0, in which there was no base-line temperature rise. Only settings in the comfort zone were considered. Friedman two-way analysis of variance was used and yielded values that did not approach significance for either sex tested separately. The existence of a time effect on voting frequency can therefore be ruled out with some confidence.

In order to obtain some appreciation of the actual frequency with which subjects adjusted their dial votes, mean setting times were calculated. The 12 boys who encountered condition 0 had a mean setting time in the comfort zone of 15·3 min., whereas the 24 boys who encountered condition 1 or condition 2 had a mean setting

time of 13·0 min. in the comfort zone and 10·9 min. outside it. For girls, the corresponding figures are 12·0, 12·7 and 12·7 min. respectively.

DISCUSSION

Temperature conditions

The present study was intended as a laboratory investigation of realistic field conditions. The choice of temperature conditions was based on field measurements in Scandinavian schools (Andersen & Lundqvist, 1966; Andersen, 1970; Erikson & Mandorff, 1967; Antoni, 1969). The constant temperatures which have traditionally been studied for their effects on thermal comfort were seldom found in practice. The typical pattern is one of rising temperature during occupation followed by a rapid decrease of temperature in the interval between lessons when windows may be opened for airing. The temperature range investigated (20–29° C.) is typical for modern school buildings in hot weather; condition 0 for buildings of high thermal capacity and conditions 1 and 2 for buildings of lighter construction with larger windows (Mandorff, 1971). The higher temperatures studied may be critical for the performance of school work. Wyon (1969) found a deterioration in the mental performance of children at 27° C. in comparison with 20 and 23·5° C., and Holmberg & Wyon (1969) found that 27 and 30° C. caused considerable deterioration in school performance in comparison with 20° C. In the present experiment subjects performed a program of tests throughout their exposure. They were thus in a work situation very similar to that in schools, and their simultaneous evaluation of thermal comfort is likely to be the more valid for this reason. However, it should be noted that temperatures judged to be comfortable are not necessarily ideal for the performance of all types of mental work (Wyon, 1970*a*, *b*).

Subjects

The subjects in the present experiment were a random sample of Danish high-school pupils. Comparison of the physical characteristics of the male subjects with published statistics for conscripts in Denmark (Statistisk Årbog, 1969) indicated that they were a typical group. Naturally, no such comparison is possible for the female subjects. However, for both sexes comparisons may be made with the data of Diem (1962) on young Americans of Western European descent. The female subjects of the present experiment did not differ from the American data, whereas the male subjects were both taller and heavier than the American average. The results of the present study are perhaps more valid for Danish schools than are corresponding American studies.

Bedford scale

The comparisons between Bedford scale results and dial vote results indicate that no systematic differences arise. As Bedford scale voting took place only at the beginning and end of each period, the spontaneous dial voting provides more data and has been used for calculations in the present work. However, the Bedford scale results depicted in Fig. 3 provide a means of relating the present results directly to previously published work on constant temperature conditions.

Dial voting

The method of spontaneous dial voting has been shown to be suitable for the study of situations where it is important not to intrude on the attention of subjects. This is the case when subjects are working or are exposed to dynamic temperature conditions, and in almost any imaginable field situation. The analysis of variance shows that the response obtained by the method of dial voting is very sensitive to small temperature changes over long periods (1° C./hr.) and to more rapid changes (4° C./hr.). Significant changes were detected even when the average response was in the comfort zone, which should therefore be regarded not as a neutral zone but rather as a zone of tolerance whose extent may very well be affected by non-thermal factors.

Sex differences

The analysis of variance revealed also significant differences between the response of males and females to changing temperatures. Male subjects in general felt hotter than female subjects at the higher temperatures, and their response increased more rapidly with temperature. The conclusion of Fanger (1970), that there is no difference between males and females in terms of thermal comfort temperatures, was reached on the basis of a literature survey and a number of experiments, all with constant temperatures close to optimal comfort and subjects who were not performing mental work. The conditions under which the present experiment was performed were therefore considerably closer to those of normal school work. It has been pointed out by Fox *et al.* (1969) that there is a physiological sex difference in the functioning of the thermoregulatory system, and the present results are in agreement with the conclusions of these authors, which were that the greatest sex differences would be found under fluctuating temperature conditions. Stolwijk (1969) has reported that men exposed to a sudden change in ambient temperature, from 30 to 50° C., tended to report discomfort earlier and to make higher estimates of the magnitude of their sensation of discomfort than did women. The present results show that this finding is true also for clothed subjects and for moderate rates of change of temperature close to the optimum. The practical consequences and the magnitude of the effect have been discussed in detail and are apparent in Fig. 7. When classes contain boys the temperatures should probably be at least 1° C. lower, and lower still if they are clothed more warmly than the girls.

Some new aspects of the thermal comfort response

Responses obtained by the method of dial voting used in the present experiment may be unambiguously divided into the three categories – too cold, comfortable and too hot – avoiding the controversy over the wording of category scales. The method of probit analysis can thus be more confidently employed, leading to the practically useful distributions of Fig. 7. The fact that dial vote responses are *spontaneous* increases their validity and allows non-thermal factors such as the passage of time to play their part in determining responses. The temporal results

provide a new basis for deciding whether a given temperature variation is acceptable; the limiting factor can be the length of time for which subjects are comfortable rather than some arbitrarily scaled estimate of how uncomfortable they become. Conventional questionnaire methods must intrude at given points in time, which in itself is likely to influence the response obtained and reduce the validity of the conclusions.

The fact that responses were spontaneous also made it possible to examine how often subjects were aware of their thermal sensations. This information cannot be obtained by questionnaire methods other than by subjective retrospection, for subjects respond only when questioned. The statistical calculations show that subjects did in fact respond more often when uncomfortable than when comfortable, providing support for the view that thermal discomfort is a distraction and can lead to deterioration of mental performance by distracting attention as well as by altering levels of arousal and effort (Wyon, 1970*a*).

CONCLUSIONS

The present study can be said to have established that spontaneous dial voting is a valid and useful method of obtaining subjective thermal responses, corresponding to conventional methods of obtaining responses and having several useful advantages over them; that thermal discomfort has a distracting effect during mental work; that 17-year-old male and female subjects respond differently to dynamic changes in thermal stress, males in general tending to feel hotter and reacting more rapidly; and that temperatures should not exceed 23° C., even for a few minutes, if no more than 5 % of normally clothed boys are to be too hot while performing mental work.

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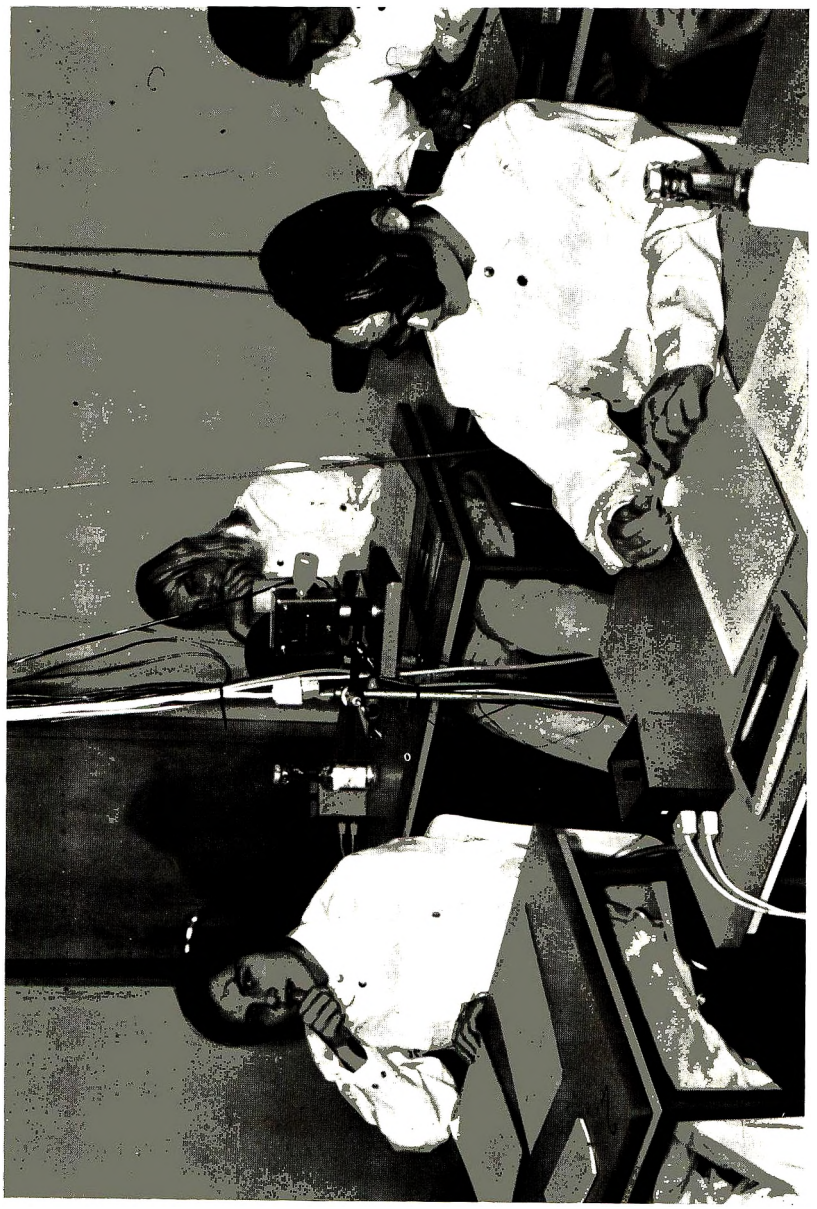

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EXPLANATION OF PLATE

Four female subjects in cotton suits (0·7 clo) in the climate chamber during an experimental session. On the table the dial vote units are seen, together with material for psychological tests. Only sedentary activities were performed.

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Survival of *Salmonella typhi* in sea-water

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SUMMARY

The bactericidal activity of natural sea-water on *S. typhi* is due to the combined effect of pH, salinity, toxic ions, presence of competitor and predator marine organisms, unidentified heat-labile toxic substances and competition for the limited food supply. Sea-water subjected to filtration and autoclaving lost the major part of its bactericidal activity. It is concluded that predators and competitors contribute significantly to the rapid death of *S. typhi* in raw sea-water. The addition of peptone decreases the bactericidal activity of sea-water. The fluctuations in the total number of competitors and predators in sea-water together with the fluctuations in the concentration of heavy metal ions may be affected by various factors. Such fluctuations are probably responsible for the variable bactericidal action of different sea-water samples.

Bathing in sewage-polluted sea-water carries with it a health risk in spite of the excessive dilution of pathogenic micro-organisms in the sea and the rapid self-purification process of sea-water. The natural purifying processes are incapable of coping with massive pollution problems and should not be relied on as the sole protection offered to users of sea-water.

INTRODUCTION

The common practice of disposing of untreated sewage and other wastes in the sea is of growing concern nowadays as it may result in the contamination of shellfish areas and bathing beaches with pathogenic agents. These agents may survive sufficiently long in sea-water to be transmitted to man either through the direct use of the water or indirectly through shellfish which have been exposed to it. Oysters and mussels concentrate bacteria suspended in the water so that, unless purified, they offer a risk of infection. The relatively low incidence of disease among swimmers in polluted areas indicates that a process of self-purification, as a result of a combination of physical, chemical and biological factors, takes place in sea-water.

The question thus arises of the fate of the enormous number of enteric bacteria which enter the coastal waters daily by way of sewage outfalls. Extensive studies have been carried out on the survival of *Escherichia coli* in sea-water (Carlucci & Pramer, 1960*a*, *b*; Carlucci, Scarpino & Pramer, 1961), but little work has been done to determine the fate of salmonellas entering bathing areas through sewage

or other means. This present study was conducted to determine the effect of pH, salinity, various chemicals, filtration, autoclaving, organic and inorganic nutrients and other factors on the bactericidal action of sea-water. In view of the prevalence of typhoid fever in the Middle East, *Salmonella typhi* was chosen as the test organism.

MATERIALS AND METHODS

Natural sea-water

Clean sea-water samples were collected, at irregular intervals, in sterile flasks from a sampling point close to the American University of Beirut Beach, about 600 m. from any sewage outfall. Most of the samples were collected in the mornings and all experiments were performed within 1 hr. of their collection. A portion of water from each collection was tested to determine its pH and salinity. The pH was determined electrometrically with a Leeds and Northrup pH meter and salinity was determined by titration with $AgNO_3$ according to the method of Volhard as cited by Vogel (1944).

Artificial sea-water

Sea-water was prepared artificially in distilled water on w/v basis according to the Constan formula for Beirut sea-water as reported by Matossian & Garabedian (1967) and according to the values reported by Tomlinson & MacLeod (1957). Likewise, solutions of single chemicals, at the same concentrations as given in the formula of Constan, were prepared in distilled water; standard analytical reagents were used for all preparations. The solutions were sterilized by autoclaving at 121° C. for 15 min.

The test organism

The test organism that was used throughout this study was a freshly isolated strain of *S. typhi* obtained from the Bacteriology Laboratory of the American University Hospital, Beirut, Lebanon. It was maintained in the lyophylized state at 4° C. until used. Standard inocula were employed routinely throughout this study. They were prepared by suspending an 18–24 hr. old nutrient agar slant culture of the test organism in sterile saline. The cells were collected by centrifugation and washed 3 times. The final cell suspension was adjusted with sterile saline to an optical density of 0·07 (transmittance 0·85) equal to a cell concentration of 10^8/ml. One ml. of this suspension was added to 100 ml. of freshly collected sea-water or other test sample in 200 ml. screw-capped bottles, yielding a final concentration of 10^6 cells/ml.

Index expression of the survival of S. typhi

The inoculated sea-water or other test samples were incubated at room temperature (25° C.) and viable counts were made immediately after inoculation and at daily intervals thereafter. For this purpose duplicate spread plates were prepared from appropriate dilutions of the inoculated sample using SS agar as the plating medium. The percentage survival after 48 hr. incubation was adopted as an index to express the surviving fractions.

RESULTS AND DISCUSSION

Several indices have been used by various investigators to express the survival of bacteria in sea-water. For example, complete survival curves were used by Orlob (1956). Others used the time required to kill 90 % of the bacteria (Vaccaro, Briggs, Carey & Ketchum, 1950), or the percentage survival after a given time (ZoBell, 1936). In our work we adopted the percentage survival after 48 hr. as an index to express the surviving fraction of the test organism and to evaluate the influence of various factors on the survival of *S. typhi* in sea-water.

The bactericidal action of sea-water was established by testing the effect of different concentrations of untreated sea-water on the survival of *S. typhi*. A 25 % concentration favoured the survival of the test organism while higher concentrations reduced it (Table 1). At 25 % concentration the effect of salinity (3·5 %) on survival of organisms is apparently abolished or somewhat reduced. Likewise, the effect of marine bacteria and other factors which normally contribute to the rapid death of the test organism are reduced because of the dilution factor.

It has been observed that bacteria survive to a greater extent in heat-sterilized than in untreated sea-water (Carlucci *et al.* 1961; Vaccaro *et al.* 1950). Our results (Table 2) confirm these findings and show that survival of the test organism in untreated sea-water was much less than in the autoclaved samples. In five of the six experiments the percentage survival was much greater than in untreated sea-water. The decreased survival of *S. typhi* in the filtered sample No. 2 may be explained by the assumption that a beneficial substance or organism was removed by filtration. It is assumed that the favourable effect of filtration and autoclaving on survival resulted from the removal or destruction of predators and competitors. These vary in number and activities from one sample to the other. This partly explains the fluctuation in the percentage survival in the six samples tested. The beneficial effect of autoclaving over filtration may be due to the presence of unidentified heat-labile toxic substances in sea-water which are destroyed by autoclaving and to the destruction of Bdellovibrio predators which are not retained by filters. Autoclaving may also disturb the ionic balance in sea-water, thus rendering these ions less bactericidal. The survival time of *S. typhi* in untreated, filtered and autoclaved sea-water samples ranged from 3 to 6, 4 to 6 and 7 to 12 days respectively.

The survival of *S. typhi* was greater in autoclaved sea-water supplemented with peptone than in untreated sea-water similarly supplemented (Table 3). In the supplemented and untreated samples peptone influenced the native population to a greater extent than the test organism. It appeared that *S. typhi* had difficulty competing successfully with the natural microflora. This indicates that competitors and predators play a primary role in limiting the utilization of peptone by *S. typhi*.

Artificial sea-water prepared according to the Constan formulation (Table 4) is less bactericidal than untreated sea-water, presumably because the former is free from predators and competitors and the latter possesses certain unidentified factors which have bactericidal properties. The bactericidal effect of artificial sea-water prepared according to the data of Tomlinson & MacLeod (1957) was more

Table 1. *Effect of various concentrations of sea-water on the survival of* S. typhi*

Concentration of sea-water (%)	Survival after 48 hr. (%)
0†	24·8
25	34·9
50	16·2
75	10·1
100	6·7

* Number of cells in the suspensions at zero time = 10^6 cells/ml.
† Deionized water.

Table 2. *Survival of* S. typhi *in untreated, filtered and autoclaved sea-water samples**

(Percentage survival after 48 hr.)

	Number of samples					
Treatment	1	2	3	4	5	6
Untreated	0·04	11·40	0·17	1·20	0·16	0·16
Filtered	14·00	1·00	12·00	5·20	1·36	6·40
Autoclaved	13·70	19·20	11·40	11·20	8·80	6·60

* Sea-water samples represent six different collections.

Table 3. *Effect of peptone on the survival of* S. typhi *in untreated and autoclaved sea-water*

	Peptone (p.p.m.)	Percentage survival after (days)			
		1	2	3	4
Untreated	0	19	10	0·012	0·006
	1	27	13	0·022	0·006
	10	30	20	0·002	0·017
	50	46·6	27	0·05	0·02
Autoclaved	0	38	21	6·9	6·3
	1	37·7	23	16	14·6
	10	39	23·5	16·7	15·0
	50	62·6	37·0	18·2	15·3

pronounced when compared with that described by Constan (Table 5). This could possibly be attributed to the additional Br^-, HCO_3^-, Sr^{+++} and H_3BO_3 present in the sea-water constituents described by Tomlinson & MacLeod (Table 4). Inorganic salts are the most potentially toxic substances in the sea. They may adversely influence the survival of bacteria by a general osmotic effect or by specific ion toxicity.

Single salts showed a bactericidal action with great difference in magnitude. Sodium chloride, potassium chloride and calcium sulphate showed a more marked

Table 4. *Composition of two artificial sea-waters*

Constituent	Constan* (%)	Tomlinson & MacLeod (%)
H_2O	96·38	95·993
NaCl	2·80	2·4
KCl	0·08	0·07
$MgCl_2$	0·37	—
$MgSO_4$	0·23	—
$CaSO_4$	0·14	—
$CaCO_3$	0·013	—
$MgCl_2.6H_2O$	—†	1·1
Na_2SO_4	—	0·4
$NaHCO_3$	—	0·02
KBr	—	0·01
$SrCl_2.6H_2O$	—	0·004
H_3BO_3	—	0·003

* As cited by Matossian. † Not included.

Table 5. *Effect of artificial sea-water and single salts on the survival of* S. typhi

Sample	Survival after 48 hr. (%)
Untreated sea-water	1·5
Artificial sea-water (Constan)	15·0
Artificial sea-water (Tomlinson & MacLeod)	0·7
Single salts	
NaCl 2·8 %	16·5
$MgCl_2$ 0·37 %	44·0
$MgSO_4$ 0·23 %	44·5
KCl 0·08 %	17·2
$CaSO_4$ 0·14%	16·0
$CaCO_3$ 0·013 %	00·0

Table 6. *Effect of pH on the survival of* S. typhi *in sea-water and in a solution of NaCl of equal salinity*

pH	Percentage survival after 48 hr. NaCl	Sea-water
5	25·60	33·50
6	4·95	4·10
7	0·67	3·30
8	0·70	1·79
9	0·00	0·35

bactericidal effect than magnesium sulphate and magnesium chloride (Table 5) Calcium carbonate solution completely abolished the survival owing to its alkaline pH (9·1). These toxic ions seemingly play an important role as bactericidal agents. The variation in the bactericidal action of natural sea-water is partly due to the fluctuation in the concentrations of toxic ions.

The pH value of sea-water as given by Harvey (1955) ranged between 7·5 and 8·5. In the present study the pH values of the sea-water samples studied were $7{\cdot}9 \pm 0{\cdot}2$ and the salinity was $3{\cdot}5 \pm 0{\cdot}1$ %. Table 6 shows that acidic pH favours the survival of *S. typhi* in contrast to alkaline pH. This suggests that acidic pH limits the survival of marine bacteria more than the test organism. The survival of *S. typhi* was higher in sea-water than in sodium chloride solution of equal salinity and pH. This may be due to the ionic balance in sea-water. A similar protective action of the balance of salts in sea-water was described by Spencer (1957).

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Inoculation of hamsters with a temperature sensitive (ts) mutant of parainfluenza 3 virus*

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SUMMARY

The multiplication pattern of a temperature-sensitive (ts) mutant of bovine parainfluenza 3 virus was studied in hamsters and compared with that of a virulent virus strain. The ts mutant was recovered regularly from the nasal mucosa and not from the lungs, whereas the virulent virus multiplied in the lungs as well as in the nasal mucosa.

The serological response induced by the mutant was comparable to that obtained after inoculation of the virulent virus.

This ts mutant may be a potential candidate for a live intranasal vaccine against bovine parainfluenza 3 infection.

INTRODUCTION

Local antibodies in the respiratory mucosa play an important role in the immunity against respiratory virus infections. The formation of such local antibodies can be induced by the intranasal administration of live, attenuated viruses which multiply locally in the nasopharyngeal mucosa. Theoretically, the safety margin of this procedure could be greatly increased by the use of viruses which have lost their capacity to multiply in the lower respiratory tract but are still capable of multiplying in the nasal mucosa. The difference which exists between the normal temperature of the nasal mucosa and that of the lungs offers an attractive approach to this problem. This approach has been investigated recently in studies in laboratory animals with temperature-sensitive (ts) mutants of various respiratory viruses (Gharpure, Wright & Chanock, 1969; Mackenzie, 1969; Mills, van Kirk, Hill & Chanock, 1969). The hamster has served as an experimental model in several of these studies (Mills *et al.* 1969; Potash *et al.* 1970; Wright, Woodend & Chanock, 1970).

In the present study we inoculated hamsters with a ts mutant of parainfluenza 3 virus of bovine origin and studied the behaviour of this mutant in comparison with that of a wild strain of the same virus.

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MATERIALS AND METHODS

Viruses

R.L.B. 103. This ts mutant was isolated in our laboratory from the 23B variant of the Umeå strain (Bakos & Dinter, 1960) of parainfluenza 3 virus; this strain was obtained originally from Dr Z. Dinter, Institute of Virology, Uppsala. The ratio of the growth at 30° C. versus the growth at 39° C. for the ts mutant was $\geqslant 10^{5.5}$ TCID 50. The virus used in the present study was grown in primary fetal bovine kidney cell cultures.

Virulent virus: WT strain. This strain was isolated in our laboratory from the lung lymph nodes of a calf with pneumonia. This strain had been passed 5 times in primary bovine kidney cell cultures. The ratio of its growth at 30° C. versus 39° C. was $\leqslant 10^{-1}$.

Cell cultures

Primary cultures of bovine fetal kidney (PBFK) were used throughout the study. The growth medium consisted of Hanks's balanced salt solution with 5% lactalbumin hydrolysate and 10% calf serum. As maintenance medium we used Eagle's medium with 2% virus-screened agamma newborn calf serum (Hyland Laboratories).

Hamsters

Syrian hamsters were obtained from our own breeding colony. They were 7–8 weeks old when used in the study.

Animal inoculation

Each hamster was inoculated intranasally with a volume of 0·1 ml. per nostril. The animals were killed at regular intervals after inoculation; heparinized blood, the lungs and the nasal turbinates were removed at the time of autopsy.

Virus isolation

The presence of virus in the organs was determined as follows: the tissues were ground in a mortar and a 1/10 (w/v) suspension was made in phosphate buffered saline (PBS) containing 200 I.U. penicillin, 200 μg. streptomycin and 100 units nystatin per ml.

The samples were centrifuged for 30 min. at 3000 rev./min. before inoculation; the blood samples were used without further treatment. Tenfold dilutions were made in PBS.

The samples were inoculated onto PBFK culture tubes, using four tubes per dilution and 0·2 ml. per tube. Two tubes were incubated at 30° C. and two others at 39° C. for 7 days. A haemadsorption test using guinea-pig erythrocytes was performed at the end of the observation period.

Table 1. *Isolation of parainfluenza 3 virus from the organs of hamsters killed at 2, 3 and 4 days after inoculation*

		Days after inoculation								
		2			3			4		
			Log titre at			Log titre at			Log titre at	
Group	Organ	Animal no.	30° C.	39° C.	Animal no.	30° C.	39° C.	Animal no.	30° C.	39° C.
A, ts mutant	Nasal mucosa	1	⩾ 2	–	4	2	–	7	⩾ 2	0
		2	0	–	5	2	0	8	2	–
		3	1	–	6	⩾ 2	–	9	2	–
	Lungs	1	–	–	4	–	–	7	–	–
		2	–	–	5	–	–	8	–	–
		3	–	–	6	–	–	9	–	–
	Blood	1	–	–	4	–	–	7	–	–
		2	NT	NT	5	–	–	8	–	–
		3	NT	NT	6	–	–	9	–	–
B, WT strain	Nasal mucosa	10	⩾ 2	⩾ 2	13	⩾ 2	⩾ 2	16	⩾ 2	⩾ 2
		11	0	1	14	⩾ 2	⩾ 2	17	⩾ 2	⩾ 2
		12	⩾ 2	⩾ 2	15	⩾ 2	2	18	⩾ 2	⩾ 2
	Lungs	10	⩾ 2	⩾ 2	13	⩾ 2	⩾ 2	16	⩾ 2	⩾ 2
		11	⩾ 2	⩾ 2	14	⩾ 2	2	17	⩾ 2	⩾ 2
		12	1	–	15	⩾ 2	1	18	⩾ 2	⩾ 2
	Blood	10	–	–	13	–	–	16	–	–
		11	NT	NT	14	–	–	17	–	–
		12	NT	NT	15	–	–	18	–	–

– = negative result. NT = not tested.

Table 2. *Haemagglutination-inhibiting antibody response in hamsters inoculated with parainfluenza 3 virus*

Group	Inoculum	HA antigen used	Days after inoculation 0 Titre	10 Titre	21 Titre
A	ts	WT	< 10	< 10	320
		ts	< 10	20–40	1280
B	WT	WT	.	40	640–1280
		ts	.	80	1280
C	None controls	WT	< 10	< 10	< 10
		ts	< 10	< 20	< 10

Haemagglutination-inhibition (HI) test

A 0·5 % guinea-pig erythrocyte suspension was used; sera from different animals taken on the same day after inoculation were pooled, inactivated at 56° C. for 30 min., treated first with an equal volume of a 25 % kaolin suspension and then with a 50 % suspension of guinea-pig erythrocytes; 1 volume of this erythrocyte suspension was added to 10 volumes of the 1/2 serum dilution. Four haemagglutinating units were used in a volume of 0·25 ml.

RESULTS

Thirty-six hamsters were divided in three groups. The first group (A) received the ts mutant; the inoculum had a titre of $10^{6·3}$ TCID 50/0·1 ml. at 30° C.; the second group (B) was inoculated with the WT strain; the titre of the inoculum was $10^{7·3}$ TCID 50/0·1 ml. at 37° C. The animals of the third group (C) were used as controls and were given Eagle's basal medium. On days 2, 3 and 4 after inoculation three animals of group A and B each were killed and their organs used for virus isolation attempts. The remaining animals were bled on days 10 and 21 and their serum examined for the presence of HI antibodies.

The results of the virus isolation studies are summarized in Table 1. No viraemia could be detected in any of the animals examined whether they were inoculated with the wild WT virus or with the ts mutant. In both inoculated groups all animals examined on the 2nd, 3rd and 4th days after inoculation harboured virus in their nasal mucosa. In group A inoculated with the ts strain all samples incubated at 30° C. were positive. No definite conclusions can be drawn from the titrations because in most cases no end-point was reached, but there may be some indication that the titre in the animals killed on the 2nd day was somewhat lower than in those killed on the 3rd and 4th days. All cultures inoculated with samples from group A and incubated at 39° C. remained negative, except for two samples where a slight haemadsorption was observed in the cultures inoculated with the undiluted material. In group B no difference was noticed between the cultures incubated at 30° and 39 °C.

Virus was recovered from the lungs of all nine animals which had received the

wild strain, whereas all attempts to isolate virus from the group inoculated with the ts mutant failed.

The results of the HI tests on the sera are shown in Table 2. Each serum pool was tested against both antigens, WT and the ts strain. No significant differences were observed. No HI antibodies were present in the pre-inoculation sera. The control animals remained seronegative throughout the observation period. In groups A and B low HI titres were demonstrated on the 10th day after inoculation. The titres had increased significantly on day 21. They were comparable in both groups.

DISCUSSION

Ts mutants can be expected to offer a wider margin of safety than viruses attenuated by other means, in those respiratory infections where the pathogenicity of the organism is related to its capacity to multiply in the lungs. In their experiments with an influenza A 2 strain in hamsters, Mills *et. al.* (1969) obtained a strong reduction of the multiplication rate of the ts mutant as opposed to the parent virus. Similar results were obtained by Wright *et. al.* (1970) with ts mutants of respiratory syncytial virus. A ts mutant of parainfluenza 1 virus showed an analogous pattern in a study in hamsters reported by Potash *et al.* (1970).

The results of our study in hamsters with parainfluenza 3 virus confirmed the theoretical expectations. The multiplication of the ts mutant was restricted to the nasal mucosa of the inoculated animals. Although the formation of local antibodies was not checked, the demonstration of circulating antibodies as a result of the intranasal application of the virus may be regarded as an indication that in all probability a local immunity was present. The circulating antibody titres observed in the group which had received the ts mutant were of the same order as in those which had received the wild strain. From the comparison of the recovery rates in cultures incubated at 30° and 39° C. we may conclude that one hamster passage had no apparent effect on the ts properties of the virus.

The behaviour of the wild strain contrasted sharply with that of the ts strain. It could be recovered from the lungs as well as from the nasal mucosa of the inoculated animals.

Live attenuated parainfluenza 3 viruses administered intranasally have been shown by Bögel & Liebelt (1964) and by Gutekunst, Paton & Volenec (1969) to produce immunity in calves. For the immunization against this disease a ts mutant might offer some additional advantages over live vaccines attenuated by other procedures. Experiments in calves, however, are required to check if the ts mutant used in our study behaves in its natural host in the same way as it does in hamsters.

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Coronavirus antibody titres in sera of healthy adults and experimentally infected volunteers

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SUMMARY

Six coronaviruses isolated in the U.S.A. have been inoculated into volunteers and all produced colds. Between 10 and 20% of infected volunteers developed heterologous antibody responses after these and other experimental infections with coronaviruses. The haemagglutination-inhibition test with the OC43 virus strain was found to detect antibody rises after infection with a variety of strains.

Studies on normal adult sera taken between 1965 and 1970 revealed a high frequency of neutralizing antibody to one strain (229E) and a frequency of HI antibody to strain OC43 which fluctuated from year to year. Complement-fixing antibodies to these two viruses were also found, revealing an apparent increase in the activity of coronaviruses in the general population of the U.K., during the winter of 1968–9.

INTRODUCTION

During the last decade a number of viruses have been isolated from cases of human upper respiratory infection which, though ether-labile, and probably containing RNA, were serologically unrelated to any known human respiratory pathogens (Tyrrell & Bynoe, 1965; Hamre & Procknow, 1966; McIntosh *et al.* 1967*a*; Tyrrell, Bynoe & Hoorn, 1968; Kapikian *et al.* 1969). It has been shown since that many of these newly isolated strains have the same characteristic morphology as that of the virus of avian infectious bronchitis (McIntosh *et al.* 1967*a*; Tyrell, 1967; Almeida & Tyrrell, 1967) and they have been classified together with murine hepatitis (Almeida & Tyrrell, 1967) and porcine transmissible gastroenteritis (Tajima, 1970) as coronaviruses. These viruses are in many cases related to each other serologically (McIntosh *et al.* 1969; Bradburne, 1970).

In previous studies of experimental and natural infections with human coronaviruses, rises in heterologous antibody titres have been found (McIntosh *et al.* 1969; Bradburne, 1970). The antigenic relationships which have been demonstrated between some human coronaviruses and mouse hepatitis viruses probably account for the presence in human sera of antibody, detected by Hartley, Rowe, Bloom & Turner in 1964, which reacts with the murine viruses in both complement-fixing and neutralization tests.

This paper deals firstly with the heterologous serological responses in human sera after experimental infection with various coronaviruses and secondly with a survey of sera of adults collected in Britain over 5 years.

Table 1. *The history of coronaviruses used for inoculation of volunteers*

Virus	Reference of isolation	Passage history (after original specimen)
229E	Hamre & Procknow (1966)	3 × OC + 1, 2 or 3 human
LP	Tyrrell *et al.* (1968)	Human only, or 4 × OC
B 814	Tyrrell & Bynoe (1965)	3 × OC or human only
EVS	Tyrrell *et al.* (1968)	3 × OC or 3 × OC + 1 human
OC 38, OC 43	McIntosh *et al.* (1967*a*)	3 × OC or 3 × OC + 1 human
No. 691 (OC 44), No. 703 (OC 48), No. 501 (OC 16), No. 663 (OC 37)	McIntosh *et al.* (1967*a*)	3 × OC

'OC' = organ culture passage.
'Human' = passage in human volunteers.

Table 2. *The production of colds in volunteers inoculated with the 'OC' strains of McIntosh*

Virus	Volunteers inoculated	No. of colds produced	Classification of cold		
			Mild	Moderate	Severe
690 (OC 43)	6	3	2	1	0
664 (OC 38)	7	7	1	5	1
691	9	6	5	0	1
703	7	7	2	4	1
501	8	2	1	1	0
663	12	10	7	2	1

METHODS

Volunteers were isolated and cared for as described elsewhere (Tyrrell, 1963). They were inoculated with various strains of coronaviruses which had been passed serially in organ cultures of human nasal or tracheal epithelium, maintained by the methods of Tyrrell & Blamire (1967). Alternatively, nasal washings taken from volunteers with colds were passaged serially to other groups of volunteers. The passage histories of the various viruses are listed in Table 1. Certain of the viruses (the 'OC' strains of McIntosh *et al.* (1967*a*)) had not previously been given to volunteers and their pathogenicities for man were unknown. Typical 'coronavirus-type' colds were induced similar to those produced by the 229E and B 814 viruses (Bradburne, Bynoe & Tyrrell, 1967). The numbers of colds induced and the severity of the disease appeared to vary with the virus strain used (Table 2), but this may be a reflexion of variation in dose of virus given and the immune state of the volunteer rather than the differences of virulence.

Serum samples were taken from volunteers on arrival at the Unit and again 3 weeks later and were stored at $-20°$ C.

Table 3. *Summary of the preparation of CF antigens used*

Virus	Antigen prepared in	Concentrated	Filtered on Sepharose 4B	Final titre (units/ml.)
229 E	L 132 cells	× 50	Yes	640
OC 43	Suckling mouse brain	—	Yes	2560
MHV_3	Suckling mouse	—	No	1280

All antigens tested against 8 units of animal antiserum.

Serological procedures

Neutralization tests. Antibody to virus 229E was estimated by 90% plaque reduction in L132 cells (Bradburne & Tyrrell, 1969).

Haemagglutination-inhibition tests (for OC 43 virus). The OC 43 strain had been isolated by McIntosh *et al.* (1967*a*) and had been shown to be indistinguishable from another isolate named OC38. The OC43 strain adapted to the brain of suckling mice (McIntosh, Becker & Chanock, 1967*b*) was used for haemagglutination inhibition tests as described by Kaye & Dowdle (1969).

Sera for use in the haemagglutination-inhibition (HI) test were diluted in phosphate buffered saline and inactivated by heating at 56° C. for 30 min. Sera and antigens were mixed in 0·025 ml. drop volumes in a microtitre system using 4 agglutinating units of antigen. This antigen was a 10% suspension of infected brain homogenized in phosphate-buffered saline (pH 7·2) which had been clarified by light centrifugation. Antigen and serum mixtures were held at ambient temperatures for 1 hr. and then 0·025 ml. of 1% fowl erythrocytes were added and allowed to settle for 50 min. at 4° C. before the tests were read.

Complement-fixation tests. Virus 229E grown in L132 cells was purified on Sepharose 4B (Pharmacia) and concentrated by dialysis; such preparations contained over 1000 antigenic units/ml. (Bradburne, 1970). OC 38–43 and MHV_3 viruses were cultivated in suckling mouse brain and 10% suspensions of infected brain tissue were used. A summary of the preparation of each antigen used is shown in Table 3.

Low doses of complement were necessary for the optimum detection of antibody and from 1·6 to 1·75 units of complement were employed, using overnight fixation at 4° C. Anticomplementary activity was removed, without impairing antibody titres, by inactivation at 65° C. for 30 min.

Before starting this survey, large batches of CF antigens for the 229E, OC43 and MHV_3 viruses were prepared, and one batch only for each virus was used throughout; each batch was subdivided and stored at −20° C. In each test 8 units of antigen were used.

RESULTS

Heterologous antibody rises in volunteer sera after experimental infection

The proportion of heterologous rises in neutralizing antibody to coronaviruses has been found to be quite small after natural (McIntosh *et al.* 1969) or experi-

Table 4. *Rising titres of HI antibody against OC* 43 *in paired sera from volunteers who developed colds after inoculation with various coronaviruses*

Virus given	No. of serum pairs tested	No. of rises (4-fold or greater) in HI antibody to OC43
229E	13	3
LP	7	0
B814	6	0
EVS	8	2
OC38+OC43*	13	6
691*	9	5
663*	12	4
501*	8	0
703*	7	0

* N.I.H., Bethesda 'OC' strains from McIntosh.

Table 5. *Rises in CF antibody titres to OC* 43, *MHV*$_3$, *and* 229 *E in paired sera from volunteers who developed colds after inoculation with various coronaviruses*

Virus given	No. of serum pairs tested	No. of 4-fold or greater rises in CF antibody to:		
		OC43	MHV_3	229E
229E	13	1	2	5
LP	7	1	2	3
B 814	6	1	0	0
EVS	8	1	1	0
OC38+OC43*	13	3	4	2
691*	9	1	1	3
663*	12	1	2	3
501*	8	0	4	2
703*	7	0	2	0

* 'OC' strains of McIntosh.

mental (Bradburne, 1970) infections. Similar studies of HI and CF antibody made after experimental infections have not been reported.

Paired sera taken from volunteers infected with all the viruses listed in Table 1 were tested by HI against OC 43. These sera were all selected from volunteers who developed colds after inoculation (see Table 2). Except for volunteers given 229E and LP, virus isolation was not attempted; however, viruses were isolated from every individual with a cold induced by 229E or LP and it is therefore likely that all these volunteers were infected.

Significant rises in the levels of HI antibody were detected mainly in volunteers given the OC 38 or OC 43 viruses, or strains nos. 691 or 663, and a few in volunteers given other strains. The results are shown in Table 4. Of 70 paired sera obtained from persons given viruses other than OC 38 and OC 43, 14 (20%) had fourfold or greater antibody rises in the HI tests.

These same sera were tested by complement-fixation against three different coronavirus antigens. To ensure that the optimum dose of complement was used

Table 6. *The numbers of heterologous antibody rises to one or more strains of coronaviruses in human volunteers*

	Volunteers given	
	229E, LP, OC38, OC43 (nos.)	Other coronaviruses (nos.)
Total pairs of sera tested	33	50
Pairs showing rises to homologous strain	19	—
Pairs showing rises to:		
1 heterologous virus	10	15
2 heterologous viruses	3	7
3 heterologous viruses	0	2

for each antigen the tests were performed at three different dilutions of complement, estimated at 1·5, 1·65 and 1·8 units/drop volume. Significant rises detected in these CF tests are summarized in Table 5. There are several examples of heterologous antibody rises.

The 229E and LP viruses have been found to be antigenically closely related (Bradburne & Tyrrell, 1969) and cross-react completely by CF tests using animal antisera. The number of significant CF antibody rises to the 229E antigen in the sera of volunteers given viruses other than 229E or LP was 10 out of 63 pairs tested (16%). The mouse hepatitis (MHV_3) antigen reacted with several sera and 18 out of 83 pairs showed significant rises in antibody titre. Some volunteers developed rises to more than one coronavirus antigen (Table 6). The actual antibody titres obtained in the HI and CF tests varied considerably amongst the 63 pairs, but generally heterologous antibody rises occurred in those with a low titre before inoculation against the heterologous virus. However, several individuals with high pre-inoculation antibody developed significant rises in antibody titres.

A survey of the frequency and titres of coronavirus antibody in normal human sera

Serum samples were taken from volunteers on arrival at the Unit. These sera represent a collection of random specimens from 'normal' healthy adults aged between 18 and 50 years and living in all parts of the U.K. Sera taken over the previous 5 years were studied. For most of this period approximately 20 sera per month were available; about 10% of these specimens represented serial samples from the same individuals, taken at minimum intervals of 6 months.

The sera were tested as follows:

(1) At a dilution of 1/20 for their capacity to neutralize 90% or more of 229E plaques produced in L 132 cell monolayers.

(2) At a dilution of 1/10 for their ability to completely abolish the agglutination of fowl erythrocytes produced by four agglutinating doses of OC 43 virus antigen.

(3) At twofold dilutions (1/10 to 1/80) for their capacity to fix at least 1 unit of complement with 4 units of either 229E or OC 43 antigens.

The results, as shown in Figs. 1 and 2, were grouped into 3-monthly periods, representing accumulated tests on a maximum of 60 sera. It can be seen in Fig.1

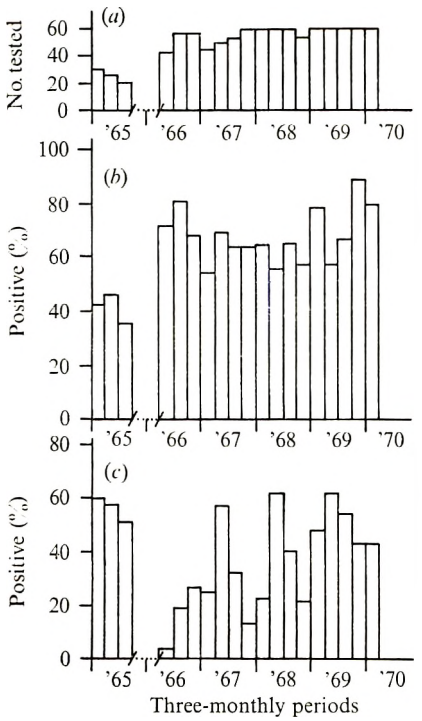

Fig. 1

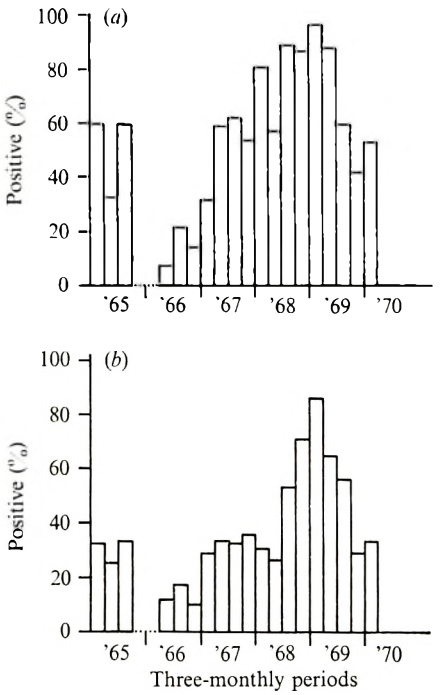

Fig. 2

Fig. 1. The numbers of normal human sera tested (*a*) and the proportion of those found to neutralize 229E virus (as a 90% plaque reduction) (*b*) when diluted to 1/20. The proportion of sera which inhibited the effects of four agglutinating doses of OC 43 virus, at a serum dilution of 1/10, are shown in the lower histogram (*c*). All results are divided up into 3-monthly periods, between 1965 and 1970. The break in the horizontal axis indicates a period when no sera were available.

Fig. 2. The proportion of normal sera (same specimens as Fig. 1) which react, at a dilution of 1/10 or greater, with 229E (*a*) or OC 43 (*b*) complement-fixing antigens.

that relatively few sera were available during 1965, and none at all during the last quarter of 1965 and the first quarter of 1966.

In Fig. 1(*b*) it is seen that of sera collected in the first three quarters of 1965, 40% neutralized 229E virus. This could reflect a deterioration of the sera after prolonged storage, but this is not borne out in the pattern of HI antibody to OC 43 virus detected during 1965 and in later years (see below). From 1966 there was a fairly high proportion of positive sera, which remained high with possible peaks in the first and last quarters of 1969.

In Fig. 1(*c*) the frequency of HI antibody against OC 43 is quite different from that of neutralizing antibody against 229E virus. There was a high frequency during 1965 and again in the first half of 1969. It was much lower in the intervening years except for prominent peaks in the second quarters of 1967 and 1968.

Fig. 2 summarizes the rates of detection of CF antibody to both 229E and OC 43 viruses. Again there are no peaks of occurrence of 229E antibody, but a low frequency in 1966, unlike that seen with neutralizing antibody. Antibodies against OC 43 likewise fell in 1966, but instead of three peaks, one peak of high incidence

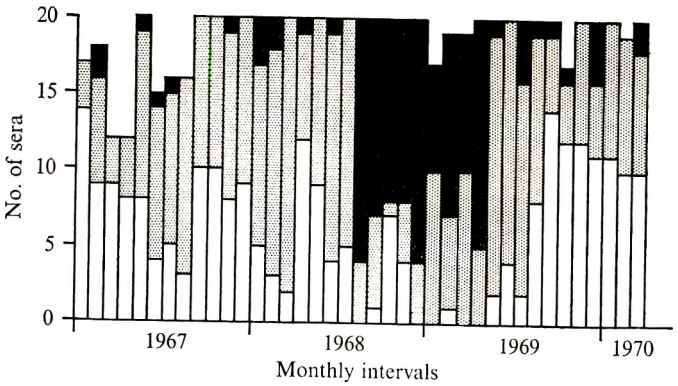

Fig. 3. The actual monthly pattern of complement-fixing antibody titres to the 229E virus in normal human sera obtained between January 1967 and March 1970. Titre range: ■, greater than 1/80; ▦, 1/10 to 1/80; □, less than 1/10.

is seen in the last two quarters of 1968 and the first three of 1969. The frequency of CF antibodies against both viruses fell towards the end of 1969. The results of CF antibody titration against the 229E antigen are analysed in more detail on a monthly basis in Fig. 3. Each column shows the number of sera without antibody, those with titres from 1/10 to 1/80, and those greater than 1/80. It can be seen that from August 1968 to April 1969 the proportion of sera with high titres of CF antibody increased considerably. Also around this period there were raised incidences of neutralizing antibody to 229E and of CF and HI antibody to OC43 virus (Figs. 1, 2).

The maximum incidence of CF antibody, found in the first quarter of 1969, were 98% for 229 E virus and 89% for OC 43 virus; these contrast with the lowest values detected in this survey of 9% for 229E and 14% for OC 43. The maximum and minimum incidences of neutralizing antibody detected against 229E virus were 91% and 37% respectively. The corresponding values for OC 43 virus HI antibody were 61% and 5%.

DISCUSSION

Heterologous responses in volunteers

The induction of the symptoms of the common cold in volunteers with the 'OC' strains of McIntosh *et al.* (1967*a*) and the passage of the infectious agents from one volunteer to another fulfils the third clause of Koch's postulates for these coronaviruses. However, for practical reasons rates of virus isolation and patterns of virus excretion from infected volunteers were not determined. Similarly, direct serological tests, using the viruses administered, were not feasible. The heterologous responses detected in the paired sera taken from experimentally infected volunteers demonstrated that some antigenic interrelationship may exist between these new virus strains, even though some of the viruses are distinct on the basis of neutralization tests (Kapikian *et al.* 1969). As yet there is no direct evidence of common or group antigens.

It is possible that the heterologous serological responses may be the result of

antigenic recall after previous exposure to a related coronavirus. Specific cross-reactions between these viruses have been demonstrated with animal antisera (McIntosh *et al.* 1969; Bradburne, 1970) using several different serological tests, and heterologous rises have been found in human sera by other workers (McIntosh *et al.* 1969; Kapikian *et al.* 1969; McIntosh *et al.* 1970). Haemagglutination-inhibition antibody rises to the OC 43 virus in sera from persons given viruses serologically similar to the 229E strain have not been demonstrated before, and this may affect attempts to separate these viruses into serological groups (Kapikian *et al.* 1969; McIntosh *et al.* 1970). The majority of the coronavirus strains seem to be capable of producing a heterologous antibody rise to mouse hepatitis virus (MHV_3) as detected by complement-fixation.

Two of the 'OC' strains of McIntosh (OC 38 and OC 43) have been reported to be identical (McIntosh *et al.* 1967*b*). Viruses designated nos. 691 and 663 also produce a high proportion of HI responses to OC 43, but were not adaptable from human tracheal organ cultures to suckling mice, unlike OC 38 and OC 43. The other viruses react with both MHV_3 and 229E antigens by complement-fixation. There is some correlation between these results and those reported by McIntosh *et al.* (1970), but in the latter studies very few heterologous rises were detected.

The incidence of antibodies in random human sera

In the light of the heterologous responses detected in volunteer sera, the antibody responses that were detected to the two coronavirus antigens used in the serological survey cannot be interpreted as being specifically due to these particular viruses. In other surveys (Kapikian *et al.* 1969; Cavallaro & Monto, 1970) the results of neutralization and complement-fixation tests have been assumed to be relatively specific to the antigens used in the serological tests. A specific assumption that the 229E and OC 43 viruses are unrelated was held by Cavallaro and Monto and that serological responses to the 229E virus represented infections with a 229E-type virus. Such an assumption may be valid while studying sera from a relatively small community, but probably does not apply to the sera studied in this report, which were taken from persons living all over the U.K. It is notable that the antibody titres, particularly in the complement-fixation test using a 229E antigen, were considerably higher, as was the rate of detection, than those reported in previous studies. This is probably the result of using a combination of concentrated CF antigens and a low (1·5–1·75 units) complement dosage.

Some of the detected variations in the incidence and titres of antibody in adult human sera were probably not significant but the larger variations may reflect genuine changes in the frequency of antibody in the general population. The occurrence of HI antibody to OC 43 virus demonstrated this particular point. The peak frequency in 1967–9 occurred in the second quarter of each year, the quarter after the 3-month period in which coronaviruses have been most often isolated from man.

Complement-fixing antibody to both viruses was not common during 1966, but increased to a peak in the winter of 1968–9, declining again over the following year. This makes it less likely that the rising frequency of antibody, detected

before 1969, was an artifact, resulting from a deterioration of sera after long-term storage. There was a significant peak in the incidence of neutralizing antibody to 229E during the first quarter of 1969. As sera positive to OC 43 by the HI test became more numerous at this time, it seems reasonable to assume that there was a wave of infection passing through the country caused by a related virus or viruses.

Epidemiological observations by Cavallaro & Monto (1970) have led them to propose that coronavirus complement-fixing activity has a relatively short half-life. In our studies it was observed that gross variations in the prevalence of antibody did not occur, but there were more marked variations in the percentage of sera with high antibody titres (see Fig. 3). This suggests that a less-sensitive CF test would only pick up such high-titre antisera, or antisera with greater avidity, resulting from recent infection with a virus closely related to that used in the CF test. It would not take into account those antisera displaying a low titre and possibly less strain-specific activity. Such sera may constitute the greater proportion of a random selection of sera taken during non-epidemic periods.

Such serological responses may not necessarily involve upper respiratory tract infection. Cavallaro & Monto (1970) obtained a significant relationship between upper respiratory disease and CF antibody responses. However, McIntosh *et al.* (1970) found a significantly negative correlation between the development of serological responses to OC 43 or MHV (strain A-59) viruses and respiratory tract disease. It is interesting that the coronavirus infections of other animals involve more generalized illnesses, and coronavirus-like particles have been detected in non-respiratory diseases of man (Zuckerman, Taylor & Almeida, 1970; Friedmann & Bird, 1969). If such viruses were related serologically to respiratory coronaviruses, they might also figure in the serological responses detected in these studies.

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Water-borne typhoid fever caused by an unusual Vi-phage type in Edinburgh

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SUMMARY

Investigation of a small series of cases of typhoid fever infected in a river between 1963 and 1970 revealed that all were caused by a single source, a carrier of a rare phage type of *Salmonella typhi*. The contamination of the river resulted from an incorrect sewage connexion with a surface water drain outfall into the river.

INTRODUCTION

Apart from occasional major epidemics, the incidence of typhoid fever in Britain is low and the actual number of cases attributable to water-borne infection is probably very small. Yet this danger cannot be overlooked. In the City of Edinburgh a total of 28 cases of typhoid fever were notified during the decade 1961–1970 (Annual Reports, 1961–70). Twenty of these probably acquired their infection abroad or during the two main epidemics occurring at Zermatt and Aberdeen in 1963 and 1964 respectively. But between 1963 and 1969, four cases occurred caused by one type of *Salmonella typhi*, Vi-phage type K 1, which is rare in the United Kingdom. The cases were apparently unassociated socially but were infected in the City, and all had a reported connexion with a local river, the Water of Leith. Whenever the cases occurred efforts were made to trace their source by examination of the river water with Moore's swabs, (Moore, 1948; 1950), but without success. During the summer of 1970 a further opportunity to investigate this problem presented itself when four more cases of infection occurred with the same phage type and the consequent investigations revealed the infective source.

HISTORY OF PRESENT OUTBREAK

The first case, an Edinburgh schoolboy aged 13 years, was admitted to the Infectious Diseases Hospital on 4 July 1970. He had been ill at home for 2 weeks with fever, headache and vomiting followed by diarrhoea, and he was diagnosed bacteriologically as typhoid fever. On inquiry at his home, it was found that the

patient and his friends had been collecting discarded bicycle parts in the Water of Leith near a muddy pool created by a rotting car seat and a fallen tree acting as a boom. Two of his friends stated independently that, while playing there together, the patient on more than one occasion had drunk the river water, probably about 7 June, but that they themselves had not done so. The home conditions of the patient were found to be very crowded, three brothers aged 19, 15 and 13 years all sleeping together on one bed settee in a small room. Bacteriological examination of 6 specimens of faeces and urine from close contacts of the patient yielded negative results; yet 24 days after the first boy's admission his brother was admitted to hospital with similar symptoms of a week's duration, and he also was shown to be suffering from typhoid fever. This boy had no connexion with the Water of Leith and had evidently been infected by his brother. This was the only secondary case in the series. However it emphasizes the need for careful surveillance of contacts of typhoid fever during the incubation period.

On 20 August a woman of 44 years who had been ill with general malaise and fever for 2 weeks was admitted to the Infectious Diseases Hospital in a confused mental state, unable at first to give a coherent history. She was an assistant in a fruit shop and had continued work until a few days before admission. On being interviewed a friend stated that they had picnicked together beside the Water of Leith on two occasions at the end of July, and that the patient had then drunk several cupfuls of the river water. She too was diagnosed bacteriologically as a case of typhoid fever.

On 17 September a boy of 11 years, one of a family of seven children, was admitted to hospital with a history of weakness and fever for 2 weeks. It was stated that he had been walking with his father and brother by the Water of Leith on 16 August. As reports had appeared in the newspapers concerning the earlier cases of typhoid fever and the association with the river, the children in this family had been told not to drink the water. Despite this the boy admitted to having done so when out of sight of the others. In his case *S. typhi* was isolated from the blood only, investigations of the faeces remaining negative throughout his illness.

These four bacteriologically proved cases completed the outbreak of 1970: three had definite association with the river, having actually drunk the water, and the fourth was a near contact of one of these in an overcrowded home.

FIELD INVESTIGATION

When the first case was diagnosed in July 1970 an investigation to trace the source of the infection was initiated by the City of Edinburgh Health Department in co-operation with the Central Microbiological Laboratories, the Lothians River Purification Board and the City Engineer's Department. This began with an intensive survey of the Water of Leith. The river is 21 miles long, rising in the Pentland Hills at a height of over 1000 ft., traverses Midlothian County and winds a tortuous course through the city, entering the estuary of the River Forth at Leith. It is popular for walks along its banks and is easily accessible for children playing and fishing. Its flow is fairly swift and it varies in depth from 1 to 3 ft. in dry

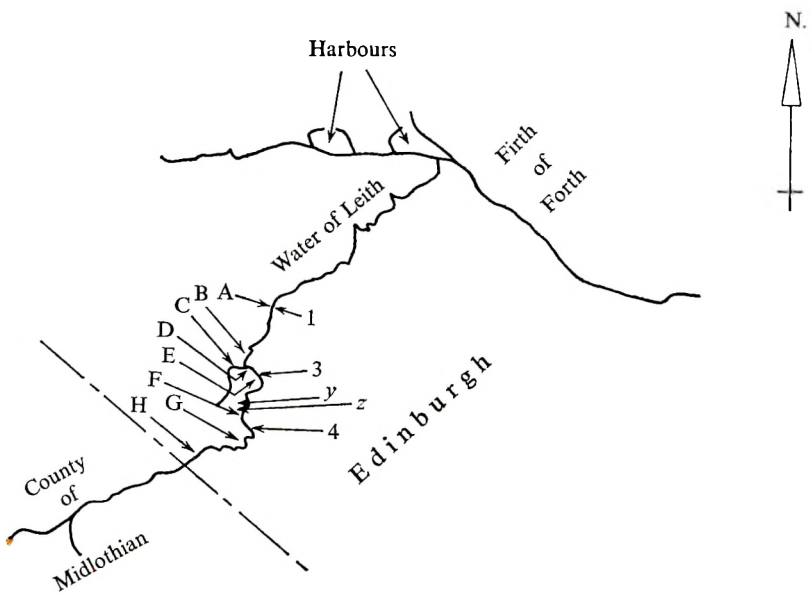

Fig. 1. Sites selected for bacteriological examination in relation to reported cases. 1, 3, 4: case connexions with river; A–H: swabs in river; *y*–*z*: swabs in sewer.

weather. The water is not used for domestic or horticultural purposes but has certain commercial uses for the mills and factories situated on its banks. For initial testing a stretch was chosen between the City of Edinburgh/Midlothian boundary and the furthest point downstream where those affected had had contact with the river, a distance of approximately $5\frac{1}{2}$ miles. Sites were selected for the positioning of Moore's swabs in the river in relation to the points 1, 3 and 4 (Fig. 1). At the same time the swabbing of adjacent sewers was instituted. The main sewer effluent from residences on both sides of the river, and from mills further upstream runs parallel to the river and in places crosses it and occasionally overflows into it. Two sites (*y* and *z*) were selected for sewer swabs – point *y* because there the main sewer crosses the river in an open channel which may on occasion overflow, while point *z* was selected because of its accessibility.

In previous years, swabs similar to those recommended by Moore had been used but were tied at the ends to form a horse shoe. For the present investigation Harrington squares cylindrically folded were tried, but were soon superseded by White's No. 1 sanitary towels (A.C.P., 1967), which were considered to be more convenient to handle, more absorbent, and therefore useful in the drains and subsidiary sewers. A thin galvanized wire (s.w.g. 20) was passed through the main substance of the towel below the stitching to support the increased strain due to the flow in the main sewer and river. The towel was folded on itself and the loops tied by 6 ft. of nylon string to withstand the friction of water, stones or rodents. Approximately 1 ft. from the loop an iron nut was attached to the nylon string. The nut acted as a sinker, to allow the swab to billow out below the surface of the water and at the same time to prevent it becoming entangled in the mud and stones on the bed of the river. The swabs were placed in the river within 3 ft. of

the bank, anchored to roots and branches of trees or to 10 in. metal tent pegs driven into the ground above the high-water level and marked by bamboo canes. In the sewers, the swabs were attached to tent pegs which were driven into the mortar joints of the brickwork or to the metal access rungs within the inspection chambers. Swabs were left in for 7 days and this practice was strictly adhered to throughout. Great care was then required in extracting the swabs as they tended to disintegrate. On removal the swab was deposited into a 2 lb. domestic Kilner jar for transport to the Central Microbiological Laboratories. The laying and collecting of the swabs was undertaken by the Inspectors from the Health Department with strict adherence to the use of protective equipment – rubber boots, disposable gloves and pliers to minimize the risk of personal infection or its spread.

S. typhi was isolated from one of the first batch of swabs received at the laboratory on 27 July. This swab was collected at the outfall of a surface water drain which discharged rain water into the river (G in Fig. 1) and which had attracted attention because it was broken and was intermittently exuding a foul-smelling effluent with occasional traces of faeces which sprayed over the bank. The opening of this pipe was situated approximately 3 ft. above the normal river level and below a metal bridge carrying a feeder sewer across the river (Pl. 1). The isolation of *S. typhi* from this swab indicated that the drain was contaminated with sewage. Repeat swabs on 3 and 10 August yielded *S. typhi* from the surface water drain only. Thus, the contamination of the river at this site was conclusively established.

Reports on the phage types of these strains isolated were received by telephone in less than 24 hr. from the Enteric Reference Laboratory of the Central Public Health Laboratory at Colindale. All belonged to Vi-phage type K 1.

As soon as the presence of the typhoid bacillus was reported measures were taken to divert the surface water into the sewer at the nearest inspection chamber. Retrograde tracing of the pathogen then began.

The surface water pipe runs through a rough bank of trees and bushes which rises steeply from the river to a street high above it which is lined on both sides with individual houses (Fig. 2). The drainage of the houses in this residential street was next explored by the City Engineer's Department. It was discovered that the foul drains from a group of houses had been connected to the surface water drain of the street. This was due to a faulty relationship between the surface water drain and the foul sewer, the former being below the other, contrary to normal practice. To narrow down the source of infection, samples of sludge were examined from the disconnecting traps between the house drain and the surface water drain at both west and east ends of this group of houses (Fig. 3). *S. typhi* was isolated from the west end sample but not from the east end sample.

Swabs were next placed at the ten access drain inspection chambers of the 12 houses as indicated in Fig. 3. The two samples at positions 9 and 10 were found to contain *S. typhi* (17 August) while the remaining eight were negative. This was confirmed by further swabbing.

It was assumed that the source of the *S. typhi* was a symptomless excreter and although the investigation of the sewage had pointed clearly to the two houses at

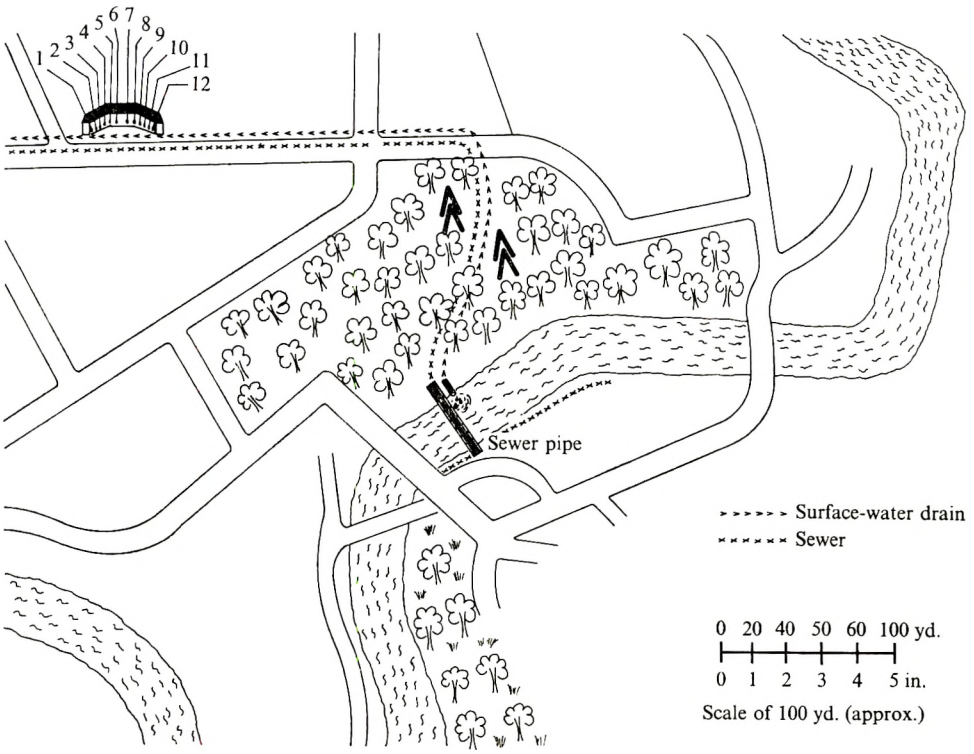

Fig. 2. Diagrammatic sketch of district showing the connexion of the sewers and drains of the houses with the river. This plan is only diagrammatic and does not give the true position of the carrier's house.

the west end of the group of twelve (P and Q, Fig. 3) it was decided to investigate the occupants of all the households to save embarrassment to anyone. Each was visited by a medical officer and invited to co-operate in this investigation to trace the source of the typhoid fever cases known to exist in the city. All agreed to participate: a history was taken in each case and arrangements were made for a series of faecal and urine specimens from each member of the households to be submitted for bacteriological examination. All specimens were negative except those from the occupant of house Q whose faecal specimens yielded *S. typhi*. The result of her Widal test suggested that she was infected with the typhoid bacillus. All strains isolated were phage typed and proved to belong to type K 1.

The whole investigation was completed within 2 months and the carrier was actually identified a fortnight before the last case was diagnosed.

BACTERIOLOGICAL METHODS

Examination of Moore's swabs

When exposed swabs were received at the laboratory double strength selenite F medium containing 0·8 % lactose was poured onto the swab until it was completely submerged. The cap was replaced and the jar was incubated at 37° C. for 24 hr. Subcultures were then made by inoculating loopfuls of the selenite on

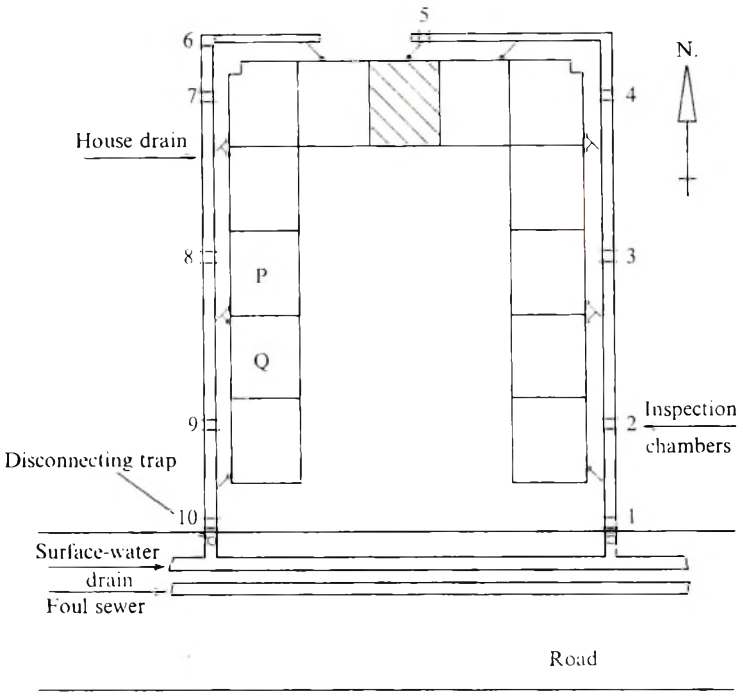

Fig. 3. Diagram of the drainage system of the 12 houses.

deoxycholate citrate agar (DCA) and MacConkey medium plates which were incubated for 48 hr. before examination. The jars were re-incubated for a further 48 hr. and subcultured to DCA and MacConkey plates as before. Non-lactose fermenting colonies were picked onto Kohn's two-tube medium (Kohn, 1954; Gillies, 1956) and after checking for purity, were identified as typhoid bacilli by routine biochemical and serological methods. Each strain was immediately sent for phage typing to the Enteric Reference Laboratory at Colindale. The positive results are recorded in chronological order in Table 1.

Examination of excreta

The final stage of the investigation was the examination of faeces and urine from the dwellers in the 12 houses. The faeces were streaked on MacConkey and DCA plates and inoculated into selenite F and Rappaport media. All media were incubated at 37° C. for 24 hr., and the two latter media were subcultured on MacConkey and DCA plates. Non-lactose fermenting colonies were again investigated as above.

Specimens of urine were centrifuged and the deposit inoculated into selenite F medium and incubated overnight at 37° C. Pale colonies from subcultures on MacConkey and DCA media were picked and investigated by the routine method. *S. typhi* type K 1 was isolated from three successive specimens of faeces from a lady aged 76 years who lived alone (Miss S). The isolations of *S. typhi* were derived from subcultures of the selenite and not from the Rappaport medium, probably because

Table 1. *Isolation of* Salmonella typhi *phage type K* 1 *in investigation of infection from Water of Leith*

Date	Source of specimen	Nature of specimen
6. vii. 70	Patient 1 (1970)	Blood and faeces
27. vii. 70	Moore's swab (No. 14) in W.o.L.*	Drain outfall into river ('G' in Fig. 1)
29. vii. 70	Patient 2	Blood and faeces
3. viii. 70	Moore's swab (27) in W.o.L.	Drain outfall into river
10. viii. 70	Moore's swab (49) in W.o.L.	Drain outfall into river
10. viii. 70	Moore's swab (46) in manhole drain	Sludge from trap (West end of street)
17. viii. 70	Moore's swab (75) in foul drain	House sewage position 9 (Fig. 3)
17. viii. 70	Moore's swab (76) in foul drain	House sewage position 10 (Fig. 3)
19. viii. 70	Moore's swab (77) in W.o.L.	Drain outfall into river
24. viii. 70	Patient 3	Blood
26. viii. 70	Patient 3	Faeces
4. ix. 70	Miss S	Faeces
7. ix. 70	Miss S	Faeces
8. ix. 70	Miss S	Faeces
17. ix. 70	Patient 4	Blood
14. x. 70	Miss S	Faeces
20. x. 70	Moore's swab (94) in foul drain	House sewage position 10 (Fig. 3)
20. x. 70	Moore's swab (95) in W.o.L.	Drain outfall into river
27. x. 70	Moore's swab (99) soil and stones (W.o.L.)	Below outfall into river
27. x. 70	Moore's swab (100) in W.o.L.	Drain outfall into river

* W.o.L., Water of Leith.

the concentration of malachite green in the latter medium is toxic for this organism (Rappaport, Konforti & Navon, 1956).

Widal tests were carried out on sera from the inhabitants of the west end group of houses. The serum of Miss S, who had no history of inoculation, showed a TH titre of 1/400, and a standard TVi titre of 1/40. The TO titre was less than 1/25. Since Miss S was symptomless, the TH and TVi titres supported the suspicion that she was a chronic carrier. A low TO reading is commonly found in typhoid carriers (Report, 1961). All other Widal tests carried out were completely negative.

Eighty-eight Moore's swabs were examined by the time the carrier was identified. Similar laboratory methods were employed in 'follow-up' swabs and faecal specimens after the sewer repair and treatment of the carrier had been undertaken. Both the sewer and the carrier remained positive until the second half of October (Table 1), but thereafter a total of a further 70 samples have proved negative.

DISCUSSION

This small outbreak has many of the characteristics of water-borne disease when the infecting water does not form part of the general supply to the community. And it has certain similarities to a small outbreak of typhoid fever described by Lendon & Mackenzie (1951) in which three children were infected with *S. typhi* in a river contaminated by typhoid-infected sewage. The cases in the present out-

break were apparently sporadic, eight known cases in 8 years, children being mostly at risk and acquiring the infection in the months of July, August and September, the ideal time of year for picnics to the river. The fact that they were all infected with a rare phage type, K 1, made a connexion between them a virtual certainty. They occurred singly in the years 1963, 1967, 1968 and 1969, but in 1970 four people were infected. The rise in the number of cases made an exhaustive investigation imperative.

Infections did not take place at the actual site of contamination of the river, possibly because access by the public at that area was difficult since the banks on both sides of the river were steep. More cases might otherwise have occurred, since the concentration of infection must have been much greater in the area of contamination.

Phage-typing was as usual of unique value in tracing the source of this outbreak. Attention had been focused on each case since 1963 as it occurred, because of the rarity of type K 1 in Britain. The type is one which is particularly associated with the Indian subcontinent, and except for Asiatic immigrants has seldom caused typhoid fever in the United Kingdom. No connexion with immigrants could be discovered in the carrier responsible for the present incident. Her history is that she has never been abroad, and had lived alone in her present house for 13 years. For 12 years before, she had been housekeeper to another lady living alone in the district who died of old age and who, as far as can be ascertained, had no connexion with the East. The carrier's father, a veterinary surgeon, had been in the Boer War and First World War and had travelled abroad subsequently to unspecified countries. She kept house for him for about 35 years before his death 25 years ago. Her sister when aged 33 years is reported to have suffered from some gall bladder condition for which she underwent an operation; within a short time she had a second operation for adhesions, from which she died. The carrier herself has apparently remained healthy throughout her life and it is impossible to judge where or when she acquired her infection. Her carrier state might never have been discovered had she not been living in a house of which the foul water drainage had been incorrectly connected with a surface water drain. Because of this, sewage containing typhoid bacilli reached a river where children played, swam and fished.

One curious historic comment completes our inquiry: according to the City Engineer's Department, no major repair to the drainage system of these houses has taken place over the years. This constructional fault has probably existed since they were built at the end of the last century. At that time the district was part of the County of Midlothian and only in 1920 was it included in the City of Edinburgh. Its houses and their drainage systems were then accepted as they stood. It was a further 50 years before the fault was demonstrated, and other cases of typhoid fever may have resulted from the coincident existence of the carrier and the incorrect sewage connexion. In the present investigation, Moore's swabs facilitated the isolation of typhoid bacilli from sewers and river, and the accuracy of phage-typing enabled us to identify cases back to 1963. Attempts are now in progress to cure the carrier; and the sewage system from these houses will be redirected so that the Water of Leith can no more be contaminated.

We thank Dr A. T. Wallace for bacteriological information regarding the patients; Dr E. S. Anderson for advice in the preparation of this paper; members of the Lothians River Purification Board and the City Engineer's Department for technical assistance; and Dr J. L. Gilloran for permission to publish.

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EXPLANATION OF PLATE

The broken drain outfall into river below the bridge supporting the feeder sewer.

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Polyethylene glycol purification of influenza virus with respect to aggregation and antigenicity

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SUMMARY

Influenza virus may be precipitated and aggregated by polyethylene glycol into clusters comprising ten to many hundred virions per aggregate. These aggregates are sparingly soluble and may be freed of contaminating polymer by washing in the appropriate buffer at room temperature or by precipitation in 30% (v/v) ethanol at subzero temperatures.

Immunogenic studies in guinea-pigs of the virus in different states of dispersion revealed that the aggregated virus is the superior antigen to the virus in the monomeric form or in the dissociated state following treatment with ether.

INTRODUCTION

It is well known that when an animal is injected with an antigen which is in a state from which it is liberated slowly (i.e. from Freund's incomplete adjuvant) the immune response of the animal is better than when injected with the antigen in its unbound or monomeric form. Influenza virus may be aggregated by polyethylene glycol (P.E.G.) M.W. 6000 into sparingly soluble clusters containing variable numbers of virions. The antigenicity of these aggregates was investigated and compared with that of the virus in its monomeric state and also when the virus was dissolved by ether.

It was observed that influenza virus could be precipitated from suspension by P.E.G. at appreciably lower polymer concentration than found by Kanarek & Tribe (1967). As high concentrations of the polymer in influenza vaccines would not meet the approval of Governmental Health agencies it stands to reason that if the content of the polymer is at a low initial value in the precipitate, removal of traces from vaccines would be more complete and more easily achieved. This study was also undertaken to explore the ways of removing this contaminant from viral concentrates.

MATERIALS AND METHODS

Virus

Two strains were used in this work, namely (1) Hong Kong A2/8/68, a strain originally obtained from the World Health Organization but passaged repeatedly in eggs in this laboratory, and (2) X 31 influenza strain, the genetic recombinant of a Hong Kong strain crossed with a PR 8 strain. These viral strains were grown in allantoic cavities of 10- to 14-day pre-incubated fertile hens' eggs and the infected fluid was harvested on the second day after inoculation.

Polyethylene glycol

Polyethylene glycol of molecular weight 6000 was used in preference to the lower or higher M.W. polymer as it has a favourable concentration–viscosity ratio (Polson *et al.* 1964). It was supplied by Shell Chemicals, Cape Town. It may be added directly to the allantoic fluid as a dry powder or as a concentrated autoclaved solution (40% by wt. in water) and was allowed to act for 1 hr. at room temperature.

Centrifuges and rotors

An MSE refrigerated centrifuge was used for freeing the infected allantoic fluid of cells and cellular debris. The No. 30 rotor and in some cases the No. 40 rotor of the Spinco preparative centrifuge Model L was employed for concentration of the finely dispersed viral aggregates. The 'thin-layer rotor' described by Polson & Stannard (1970) was employed for preparation of influenza virions in the monomeric state in a concentrated form, freed of most of the extraneous protein. (For the purpose of this paper, spontaneous loose viral aggregation is disregarded in the use of the term 'monomeric state'.)

Analytical ultracentrifuge

Several of the preparations, namely the influenza virus obtained by thin-layer rotor centrifugation and fractions precipitated by 4% P.E.G., were centrifuged in a Spinco Model E ultracentrifuge to establish whether the majority of the virus particles were present as monomers or as aggregates.

Electron microscope

The various influenza virus preparations were negatively stained with phosphotungstic acid and examined in a Siemens Elmishop Model 1A.

Antigenicity tests in guinea-pigs

Comparable groups of six guinea-pigs were used for testing each of the antigen preparations. The animals received a single intramuscular injection of formaldehyde-treated virus material without adjuvant and were bled by heart puncture before inoculation and at 3 and 7 weeks after inoculation. Antigenic potency was assessed by haemagglutination inhibition tests with fowl red blood cells, using four haemagglutinating doses of virus and reading a 100% end-point after 1 hr. at room temperature in a microtitration system.

EXPERIMENTAL

A. *Effect of storage and* P.E.G. *concentration on precipitation of virus*

After storage at 4° C. for 20 hr. the infected allantoic fluid was subdivided into eight equal portions, each of which was treated with an appropriate weight of P.E.G. powder. They were then centrifuged at 500–800*g* for 20 min. Portions of the supernatant fluids were then re-centrifuged at 1500–1900*g* for the same time. Samples of all the supernatants were kept and assayed for haemagglutinin content.

The above experiment was repeated in parallel upon infected allantoic fluid from the same original pool but which had been stored at 37° C. for 20 hr. The object of this test was to investigate the effect of storage at 37° C. upon the precipitability of the haemagglutinin by P.E.G. If there was an appreciable amount of free haemagglutinin resulting from dissociation of virus, these smaller particles would fail to precipitate at concentrations of P.E.G. which would precipitate the intact large virus particles (Juckes, 1971).

It was established that precipitation with 2 % P.E.G. followed by centrifugation at 1500*g* for 20 min. would remove approximately 90 % of the H.A. activity, and several experiments were then conducted to determine the yield of the haemagglutinating virus. This was done by repeated precipitation of the resuspended virus precipitate from diminishing volumes of dispersion medium.

Prior treatment of clarified infected allantoic fluid with 0·1 % formaldehyde did not materially alter the removal of haemagglutinin with P.E.G.

B. *Sequential precipitation with* 0·6 % *and* 4·0 % P.E.G.

The infected allantoic fluid was treated with 0·6 % P.E.G. and the resulting faint precipitate was centrifuged out of suspension at 1500–1900*g* for 20 min. and examined in the electron microscope. The concentration of P.E.G. in the supernatant fluid was then increased to 4 % and the new precipitate was centrifuged out and examined as before. The angle head of the preparative Spinco centrifuge was used and it was found that the virus precipitate failed to form a pellet but adhered to the surface of the centrifuge tubes remote from the centre of rotation. The material on the wall was also subjected to electron microscopy. The object of this experiment was to establish if it was possible to separate filamentous and giant forms from the smaller round or 'doughnut' forms of the virus or to eliminate undesirable material such as mycoplasma-like bodies and coarse non-specific proteinaceous substances from the virus suspension.

C. *Thin-layer rotor concentration*

The virus was concentrated in the thin-layer rotor at 1200–1400*g* for 60 min. The virus contained in 40 ml. infected allantoic fluid was sedimented to the extent of 90 % or more into approximately 1 ml. of fluid. This was estimated by loss of haemagglutinating activity in the supernatant fluid (SNF).

On 'washing' the virus by diluting with phosphate-buffered saline (PBS) at

pH 7·3 and a second cycle of centrifugation the associated proteins were reduced well below the level at which they could be detected by ultracentrifugation in the Model E.

D. *Antigenicity of Hong Kong A* 2/8/68 *virus precipitated by* P.E.G. *from infected allantoic fluid*

Thirty ml. of allantoic fluid infected with the strain of Hong Kong virus was treated with 4% P.E.G. added as a 40% autoclaved solution and the precipitate obtained at 900–1200***g*** for 60 min. was resuspended in 2·0 ml. of PBS to form what was termed 'aggregated antigen (*a*)'. An equal volume of the same pool of allantoic fluid was concentrated at 1200–1400***g*** for 2 hr. in the thin-layer rotor to give a final concentrate diluted to 2 ml., forming the 'monomeric antigen (*b*)'. Both antigen preparations were held overnight at 4° C. with 0·05% formaldehyde. The absence of infectious virus was confirmed, as in all experiments, by failure to induce haemagglutinin formation in embryonated hens' eggs. Each antigen was inoculated into six guinea-pigs, the animals receiving each a single intramuscular injection of 0·1 ml. of the antigen.

E. *Antigenicity of X* 31 *recombinant strain of influenza virus precipitated by* P.E.G. *from partly purified suspension*

The virus, partly purified and concentrated by clarification, dialyses against five volumes of buffer overnight at 4° C., pervaporation in cellophane to one-fifth volume and concentration in the thin-layer rotor with a wash cycle as described, was divided into three equal portions.

(*a*) The first portion was diluted eightfold with PBS and precipitated with 4% P.E.G. added as a powder, centrifuged from the supernatant (900–1200***g*** for 60 min.), resuspended in PBS and centrifuged as before; the final deposit was resuspended in PBS to the original starting volume of this portion. After 0·1% formaldehyde inactivation this formed aggregated antigen (*a*).

(*b*) The second portion received no treatment other than an inactivation with 0·1% formaldehyde. This formed the monomeric antigen (*b*).

(*c*) The third was disrupted by shaking with ether for 5 hr. at 4° C. after Brandon, Timm, Quim & McLean (1967). After removing the ether, restoring the volume and treating with 0·1% formaldehyde the dissociated antigen (*c*) was formed.

In this experiment each guinea-pig was inoculated with 1·0 ml. of a 1/2 dilution of antigen. It will be seen that all experimental animals received equal amounts of virus antigen as calculated on the basis of equivalent dilutions of the common starting material (assuming no losses).

RESULTS

In Fig. 1 the remaining haemagglutinin activity of the supernatant is related to the P.E.G. concentration on material stored at 4° C. for 20 hr. The two curves represent respectively the results achieved by centrifuging at 650***g*** for 20 min. and

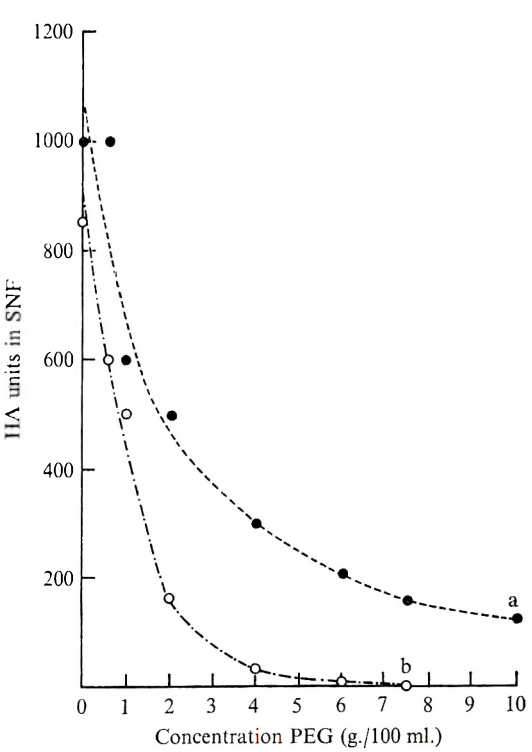


Fig. 1

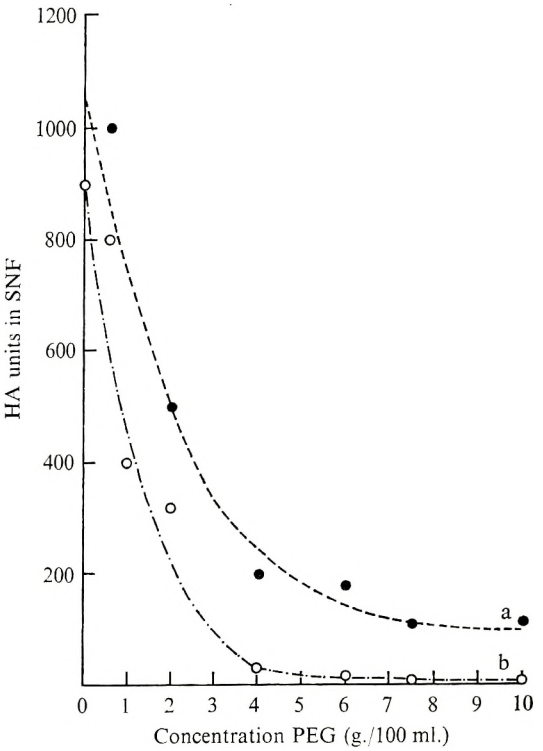


Fig. 2

Fig. 1. Haemagglutinating activity (50 % end-points) remaining in the supernatant after centrifugation of the precipitate formed on addition of P.E.G. to different concentrations. The infected allantoic fluid was stored for 20 hr. at 4° C. prior to precipitation. (*a*) After centrifugation at 500*g* for 20 min. (*b*) After centrifugation at 1500*g* for 20 min.

Fig. 2. Haemagglutinating activity (50 % end-points) remaining in the supernatant fluid after centrifugation of the precipitate formed on addition of P.E.G. to different concentrations. The infected allantoic fluid was stored for 20 hr. at 37° C. prior to precipitation. (*a*) After centrifugation at 500*g* for 20 min. (*b*) After centrifugation at 1500*g* for 20 min.

at 1200*g* for the same time, as obtained in Expt. A. In Fig. 2 is presented similar 'precipitation' curves as in Fig. 1 but here obtained with infected allantoic fluid stored at 37° C. for 20 hr. The Hong Kong A 2/8/68 and X 31 strains behaved alike in P.E.G. precipitation. The results on the recovery of virus in terms of H.A. activity are presented in Table 1. The data are from two independent experiments.

By interpolation in Figs. 1 and 2 it is clear that approximately 90 % of the H.A. activity is removable from the SNF by 2 % P.E.G. but the H.A. (titre increase)/(concentration factor) ratio is appreciably lower, indicating that the majority of virus particles are in a state in which they cannot participate individually in the H.A. reaction. This would be the case if they are forced into clusters by the P.E.G.

By initial treatment, as in experiment B, with 0·6 % P.E.G. followed by centrifugation, much extraneous material, some viral aggregates (possibly previously present) and mycoplasma-like particles (which were sometimes present in the egg

Table 1. *Recovery of virus following precipitation with* 2% P.E.G.

(Precipitates spun out at 500*g* for 20 min., resuspended and precipitated from decreasing volumes of medium using 2 % P.E.G.)

Expt.	Volume (ml.)	H.A. titre	Concentration factor	H.A. titre increase
1	200	640	—	—
	10	5,120	× 20	× 8
	2·5	10,240	× 80	× 16
2	100	1,280		
	20	2,560	× 5	× 2
	2	20,480	× 50	× 16

stock (Plate 1, fig. 1) were removed. The loss of haemagglutinating activity in the supernatant resulting from such treatment was 10–15%. Separation of filamentous from round particles was suggestive but requires further investigation.

Electron microscopy yielded informative results. Plate 1, fig. 2 is an electron micrograph of Hong Kong A 2/8/68 after concentration and purification in the thin-layer rotor. This preparation upon analytical ultracentrifugation in the Model E showed a sedimenting boundary which broadened rapidly but from which an average sedimentation coefficient of 690 Svedberg units could be calculated. The electron micrograph (Plate 2, fig. 3) was of the same pool of virus-infected allantoic fluid but precipitated with 4% P.E.G. The intimate interlocking and binding of the virions forming large aggregates which did not disperse to the monomeric state was confirmed by centrifugation in the Model E. This precipitate obtained with 4% P.E.G. was centrifuged under the same conditions as the virus in its monomeric state but it failed to show any sedimenting boundary as the material had already sedimented out when the required rotor velocity had been reached. Electron micrographs of the X 31 strain of influenza virus concentrated by thin-layer centrifugation and by precipitation with 4% P.E.G. are depicted in Plate 2, fig. 4 and Plate 3, fig. 5 respectively.

The results of the antigenicity studies D and E are presented in Tables 2 and 3 respectively.

As there was some variation in the response of the individual animals in the different groups, the haemagglutination-inhibiting activity is presented as a mean value. The occasional serum from control non-injected animals similarly bled showed slight haemagglutination inhibition at 1/10 dilution but no higher. This was not considered significant.

Clearly the 'aggregated' antigen in Expt. D produced a better response than the 'monomeric' antigen.

It must be remembered that in Expt. D an 'aggregated' antigen had been subjected to a series of further procedures as compared with the control 'monomeric' antigen but that for the purpose of administering equivalent masses of antigenic material losses during such procedures were ignored. The 'aggregated' antigen was thus handicapped from the start. However, it still produced at least

Table 2. *Experiment D: immunization of guinea-pigs with Hong Kong A* 2/8/68 *inactivated with formaldehyde*

Antigen*	HA titre after concentration	Guinea-pig serum HAI titres† Before immunization	3 weeks after immunization	7 weeks after immunization
(*a*) Aggregated	3,200	< 10	37·0	13·3
(*b*) Monomeric	12,000–25,000	< 10	12·5	6·6

* Thirty ml. infected allantoic fluid, HA titre 2560. (*a*) Precipitated with 4 % polyethylene glycol, resuspended in 2 ml. buffer. (*b*) Concentrated to 2 ml. in the thin layer rotor.

† Six guinea-pigs inoculated with each antigen. Titres are geometric means.

Table 3. *Experiment E: immunization of guinea-pigs with X* 31 *recombinant strain inactivated with formaldehyde*

Antigen*	HA titre after concentration and/or dissociation	Guinea-pig serum HAI titres† Before immunization	3 weeks after immunization	7 weeks after immunization
(*a*) Aggregated	400	< 10	50	27
(*b*) Monomeric	1600	< 10	40	30
(*c*) Ether dissociated	800–1600	< 10	18‡	10

* 400 ml. infected allantoic fluid concentrated and purified by procedures described in section E (text) and divided into three portions. Portion (*a*) was aggregated with 4 % polyethylene glycol, portion (*b*) was used in the monomeric state, and portion (c) was dissociated with ether.

† Six guinea-pigs inoculated with each antigen. Titres are geometric means.

‡ Three of the six guinea-pigs died, the values after 3 weeks and 7 weeks therefore not statistically significant.

as good a response as, if not better than, the control. Furthermore, the 'aggregated' antigen had but one quarter of the haemagglutinin titre of the control.

The poor response to ether-treated virus antigen was disappointing in the light of the generally rather favourable antibody responses reported in the literature.

The authors feel that the ether treatment may have been far too harsh, fragmenting the material excessively and the significance of the result is questionable in view of the small number of animals which survived the test.

Removal of traces of P.E.G. *from the purified virus*

In view of the importance of the removal of traces of P.E.G. and extraneous egg proteins from the purified virus vaccines, the precipitate or aggregates formed by the displacement action of P.E.G. must be considered. When an influenza virus suspension is treated with the polymer the ensuing precipitate is composed of aggregates of virus particles ranging from one particle to several hundred. The

particle size distribution of the individual aggregates also depends upon the initial concentration of the virus. Thus when a suspension which had a small number of monomeric particles per unit volume is precipitated, the aggregates formed are composed of small numbers of virions. When the concentration of the infectious agent is high, the precipitate with P.E.G. is composed mainly of clusters of large numbers of monomeric units. If we regard the individual aggregates as approximately spherical in shape an important conclusion may be drawn regarding their solubility. Thus thermodynamic theory predicts that the escaping tendency or solubility from curved surfaces is inversely proportional to the radius of the curved body.

If we regard the solubility as being directly proportional to the escaping tendency or difference in molar free energy ΔF the quantitative relationship

$$\Delta F = \frac{2\gamma V}{r}$$

(see Lewis & Randal, 1923) would explain the behaviour of the clusters of particles in suspension. In this equation γ is the surface tension, V the molar volume and r the radius of the curved surface. The entities γ and V are the same for all the aggregates, therefore the radius r is the only factor which would influence the solubility of the precipitate. If a precipitate is composed of clumps of different radii it may be expected that the smaller particles will dissolve and be re-aggregated into the larger units. This transfer of monomeric particles from the small to the larger aggregates must be time-dependent and the final state of equilibrium may only be reached after a period of an hour or longer.

This appears to be the case as it is necessary, when doing precipitation of influenza virus with P.E.G., to leave the precipitated virus for a period of 1–2 hr. before further treatment. During this period the precipitated suspension, being transparent initially, becomes opaque, indicating the formation of larger aggregates. It would therefore appear that there is a very complex equilibrium condition existing amongst the various size aggregates in the suspension of the virus precipitate. It may thus be expected that an increase in the monomeric form in the suspension would favour the production of coarse precipitates which may be removed from suspension by centrifugation at low rotor velocities. Similarly by decreasing the number of monomeric forms of the virus or by performing the precipitation on infected fluid of low H.A. titre, fine precipitates will form. These will not change noticeably in degree of dispersion with time and would require higher rotor velocities for their removal from suspension.

As the degree of dispersion is dependent upon the amount of virus initially present and as the solubility of the clumps is an inverse function of their size, these facts must be considered when traces of P.E.G. are removed from a suspension. The clumps of virus have a limited solubility and the contaminating P.E.G. is completely soluble and by suspending the virus precipitate in buffer of a volume three times that of the precipitated virus suspension very little virus in the monomeric state is left in the SNF after centrifugation. By repeating the 'washing' three times the P.E.G. content may be reduced to levels at which the polymer is no longer detect-

able but retaining most of the virus in an aggregated state, indicating a concentration of less than 0·01% P.E.G. which can be detected by Nessler's reagent (Polson *et al.* 1964).

It is our experience that when the suspension is diluted in the ratio of 1 part concentrated precipitate to 99 parts of diluent the virus precipitate dissolves slowly. Washing the precipitate with large volumes of buffer is therefore not recommended.

In addition to the differential removal of P.E.G. by washing the method also ensures the removal of extraneous egg protein which co-precipitated with the influenza virus particles.

If complete recovery of the virus antigen is desired by the differential removal of the contaminating P.E.G., the suspension of the precipitate may be cooled to approximately 0° C. and then mixed with 30% ethanol in saline at −20° C. and centrifuged. Under these conditions the virus precipitate is completely insoluble while the traces of contaminating P.E.G. dissolve in the ethanol-saline. This method is termed the cryoethanol procedure. A similar method for the removal of P.E.G. from protein fractions has been described by Polson *et al.* (1964).

Electron micrographs of the virus precipitates after an additional cryoethanol treatment indicate that approximately 10–15% of the virus particles had apparently lost their nucleo-proteins. It would appear that the monomeric and filamentous forms of the virus were more resistant to ethanol than the large or giant forms which seemed to have lost their nucleoproteins, while the doughnut and filamentous forms appeared to be intact as shown in the electron micrograph (Plate 3, fig. 6).

DISCUSSION AND CONCLUSIONS

Influenza virus may be precipitated with polyethylene glycol at polymer concentrations appreciably lower than those used by Kanarek & Tribe (1967). The precipitation curves after storage at 4° and 37° C. for 20 hr. are essentially similar and it is clear that 90% or more of the haemagglutinating material, probably intact virus or, less likely, free haemagglutinin, was precipitated by P.E.G. at polymer concentrations between 2% and 4%. The shape of the curves indicate that the material being precipitated was of an inhomogeneous nature. Both the Hong Kong A 2/8/68 and the X 31 strains behaved identically in their precipitation with P.E.G. The precipitates which formed were fine aggregates of virus particles ranging from a few, i.e. 10, to more than 100 per cluster as revealed by electron microscopy. The smaller aggregates required higher centrifugal forces to sediment them from suspension than coarse protein precipitates ordinarily produced by P.E.G. These forces (1500–1900*g*) were nevertheless appreciably lower than those required for sedimenting the monomeric form of the virus into a pellet.

All attempts to disperse the P.E.G. virus aggregates into monomeric forms without severe damage to the virions failed. It was therefore possible to wash the precipitated virus with small volumes of suitable buffer and thus free it of traces of P.E.G. and extraneous proteins with the loss of only a small amount of virus. The haemagglutinating activity of the resuspended precipitate was one-quarter to one-

fifth of the original untreated allantoic fluid and the supernatant fluids had activities which were of the order of 1 % to 5 % of the original. This indicated that the influenza virus either had been damaged, at least in respect of its haemagglutinin, or had associated into aggregates. As P.E.G. is a relatively inert material which precipitates substances from solution by a replacement mechanism as discussed by Juckes (1971) it was concluded that the individual virus particles were not significantly altered but that they were clumped together, a conclusion supported by their appearance in the electron microscope and their behaviour in the analytical ultracentrifuge.

On comparing the electron micrographs of the virus aggregated by P.E.G. with those of the virus in the monomeric state it would appear that the haemagglutinin subunits of adjacent virions are more closely interlocked in the P.E.G. aggregated material than in material concentrated in the thin-layer rotor. This interlocking, induced by the P.E.G., may be responsible for the observed failure of such aggregates to disperse. It is of interest to note that Kanarek & Tribe (1967) found complete quantitative recovery of infectivity and an apparent increase in haemagglutinin activity in some cases after treatment of dilute myxovirus suspensions with P.E.G. This phenomenon they attributed to disaggregation of agglutinated virus particles. Complete dispersion of the aggregates is thus implied. This is in contrast with the work described in the present paper on closely related viruses but at considerably higher concentrations.

Immunization experiments with the virus in various states of aggregation indicate that the virus when aggregated is more immunogenic than the virus in the monomeric state. It is suggested that the relative stability and indispersibility of the aggregates may account for the enhanced antigenicity as assessed by the production of haemagglutination-inhibiting antibody in guinea-pigs. In the present context it is clear that the haemagglutinating activity tests are not applicable in assessing antigenic content or antigenicity of an aggregated antigen preparation.

Very recently Larin & Gallimore (1971) have shown that the relationship between haemagglutinating activity and immunogenicity of an influenza antigen is merely coincidental; this inference is supported in the present work.

The results suggest that P.E.G. is of value in viral vaccine production. Coarse debris including mycoplasmas may be removed by low-speed centrifugation in the presence of very low concentrations of P.E.G. An increase in P.E.G. concentration may cause aggregation of virus particles, such aggregates being easily concentrated and washed free of contaminants by low-speed centrifugation, and such aggregates constitute good antigenic material. Their inactivation poses no problem since the virus may be inactivated before precipitation with P.E.G.

It is suggested that the P.E.G. precipitation followed by washing the precipitate with buffer to free it of extraneous protein and traces of P.E.G. be used as an alternative method to the very costly technique of zonal centrifugation of Gerin & Anderson (1969) for the purification of influenza and other viruses in the production of vaccines for human and animal immunization.

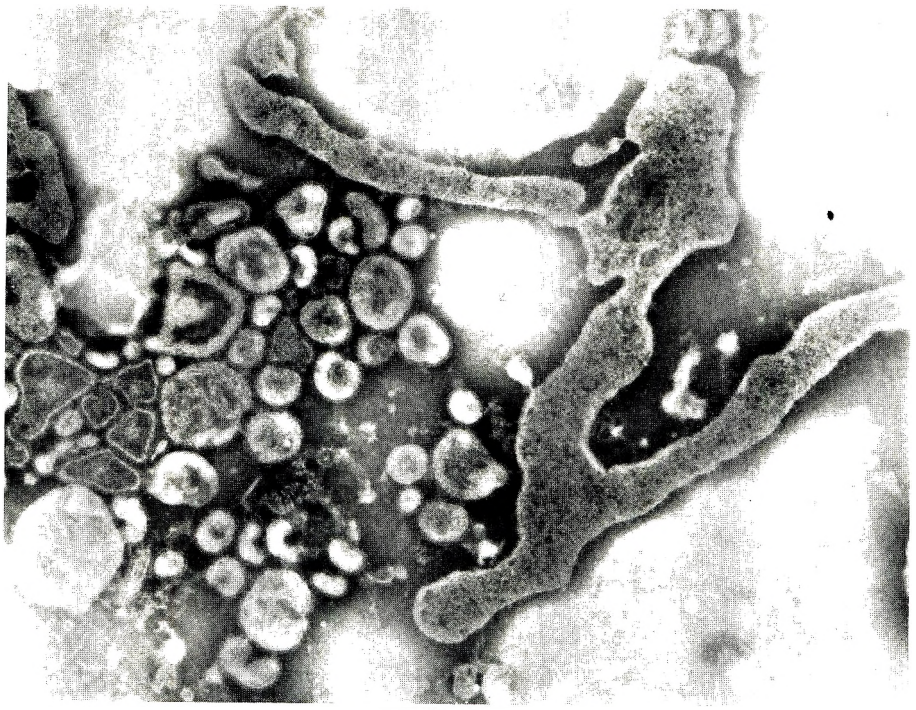


Fig. 1

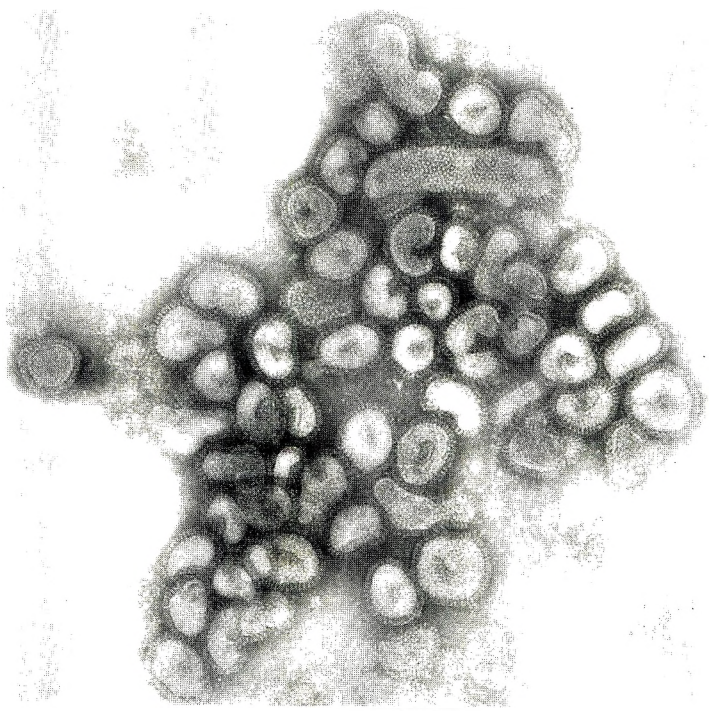


Fig. 2

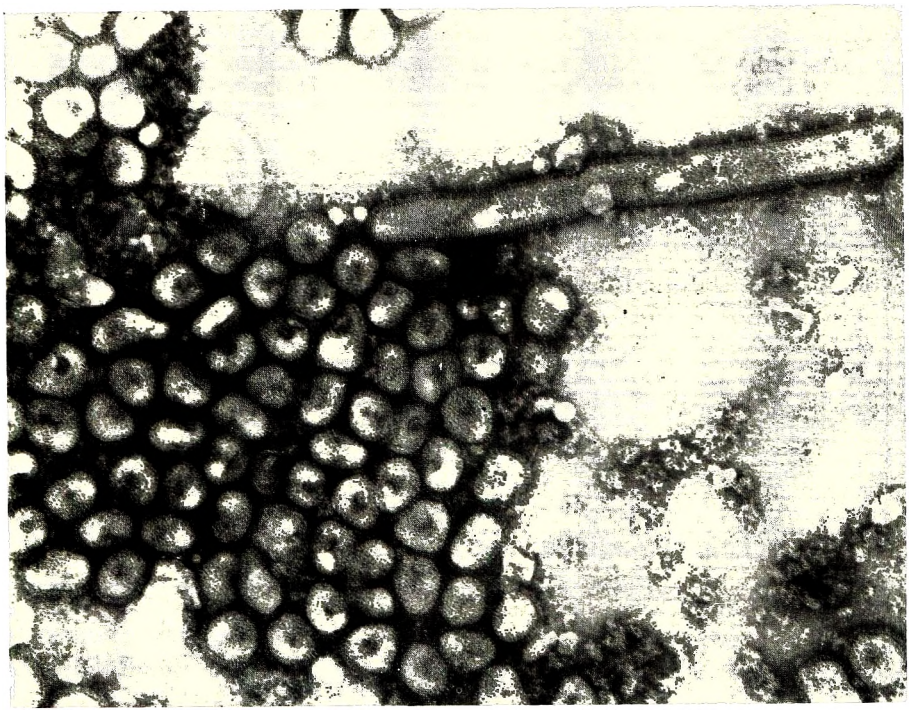
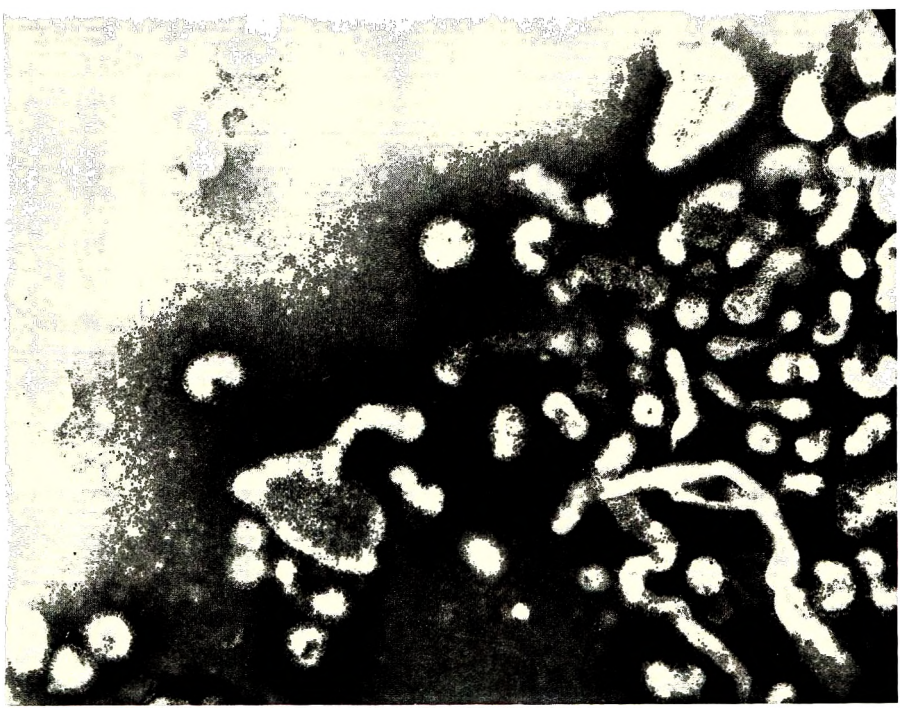


Fig. 3



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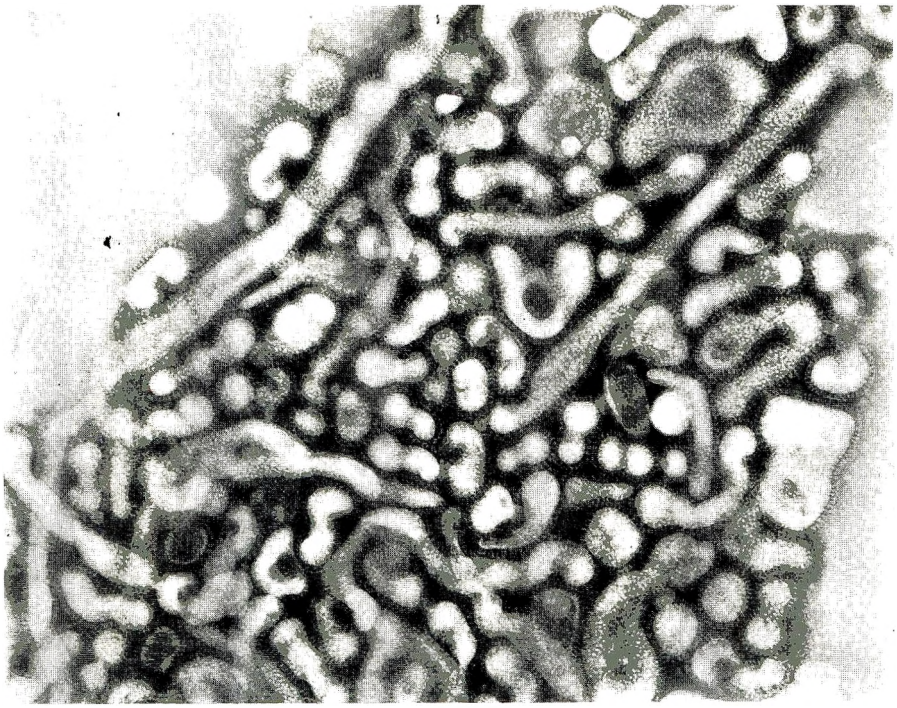

Fig. 5

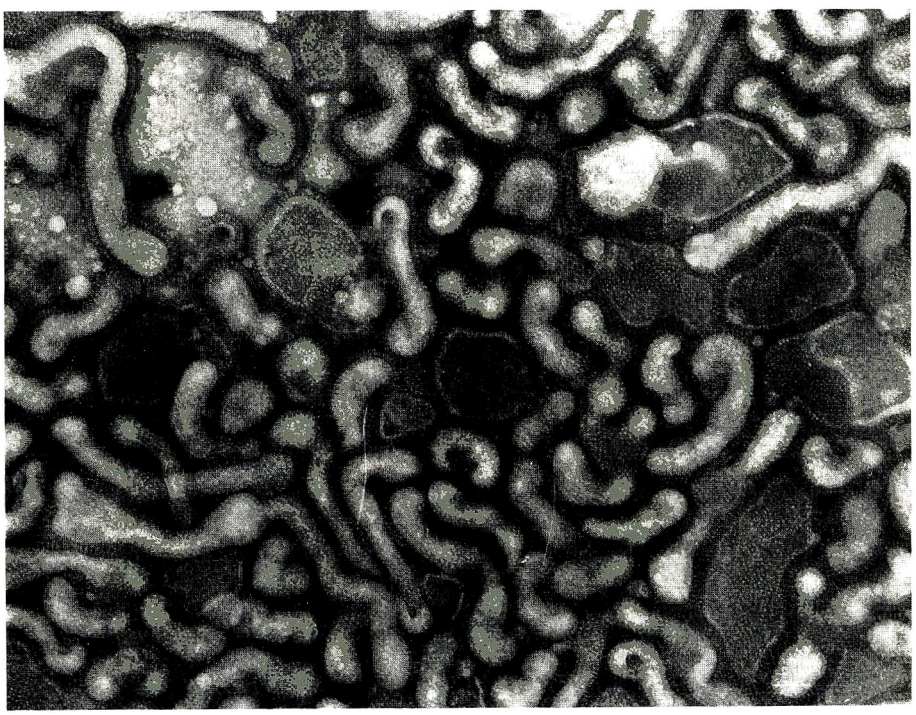

Fig. 6

Grateful acknowledgements are made to Miss Linda Stannard for the electron micrographs; to Professor A. Kipps for his interest in the work and to Mr A. M. Schady of the S.A. Inventions Development Corporation for his encouragement and criticism. The opportunity of one of the authors (A.P.) to work in the Laboratories of Messrs Parke-Davis (Detroit) is appreciated.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Electron micrograph of fraction obtained on precipitation of infected allantoic fluid with 0·6 % P.E.G. Note the large mycoplasm-like particle. × 60,000.

Fig. 2. Electron micrograph of Hong Kong A 2/8/68 concentrated with the thin-layer rotor at 500*g* for 60 min. Note the loose association of the virus particles. (Compare Fig. 3.) × 60,000.

Plate 2

Fig. 3. Electron micrograph of Hong Kong A 2/8/68 influenza virus precipitated with 4 % P.E.G. Note the close association of the virus particles. (Compare Fig. 4.) × 60,000.

Fig. 4. Electron micrograph of strain X 31 influenza virus concentrated by thin-layer ultracentrifugation at 1500*g* for 60 min. Note the loose association of the virus particles. (Compare Fig. 5.) × 60,000.

Plate 3

Fig. 5. Electron micrograph of strain X 31 influenza virus concentrated by precipitation with 4 % P.E.G. Note the close association of the virus particles. × 60,000.

Fig. 6. Electron micrograph of influenza X 31 initially precipitated with 4 % P.E.G. then resuspended in 4 volumes of buffer and finally reprecipitated in 30 % ethanol at − 20° C. Note that the smaller virus particles are intact and that the larger particles seem to be devoid of internal components. × 60,000.

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Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination reactions

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SUMMARY

Serum proteins from *Mycoplasma gallisepticum* culture medium could be detected on the organisms as a result of incubation at low pH. Only certain of the serum proteins, including IgG and IgM, were found, and the adsorption appears to depress the haemagglutinating activity of the organisms. There was no obvious effect on slide agglutination (SA) sensitivity but an incidental finding was that brief acid treatment enhanced the SA sensitivity of an antigen prepared from a young culture.

INTRODUCTION

The adsorption of protein to cell surfaces is a well-recognized phenomenon (King, 1968). In many cases the reaction appears to depend on specific protein reacting with specific cells, e.g. the fixation of protein hormones to sensitive cells, and the adsorption of cytophilic antibody to macrophages. In other cases, however, adsorption is less specific, such as the adsorption of protein to tanned red blood cells.

Hamburger, Pious & Mills (1963) demonstrated that mammalian cells in tissue culture adsorbed serum protein components from the growth medium, and, after some initial studies by Smith, Dunlop & Strout (1966), Jordan & Kulasegaram (1968) reported that *Mycoplasma gallisepticum* organisms also picked up proteins from their growth medium. In neither case could the contaminating proteins be removed by extensive washing, and inoculation of the washed cells or mycoplasmas into animals resulted in the production of antibody to the adsorbed proteins. Thus, false results may be obtained in serological tests using mycoplasmas if reactions between adsorbed proteins and their homologous antibodies should occur. Adsorbed proteins may also cause complications that are less readily recognized than the addition of new antigenic determinants. For example they may physically mask some of the existing determinants and thus reduce the specific reactivity of the mycoplasma antigens. These effects could be of considerable importance when such antigens are used for the serological diagnosis of *M. gallisepticum* infection in poultry flocks.

This communication describes (*a*) a study of the conditions of culture of *M. gallisepticum* that give rise to adsorption of medium proteins, (*b*) preliminary identification of the adsorbed proteins, and (*c*) a study of their effect on *M. gallisepticum* antigens in agglutination and haemagglutination tests.

MATERIALS AND METHODS

M. gallisepticum *strain*

A 514 originally obtained from Dr H. Chu, University of Cambridge, passaged numerous times in culture in this laboratory, was used.

Mycoplasma growth media

Broth medium (*BM*). This was prepared as described by Bradbury & Jordan (1971*a*) with 15% (v/v) heat-inactivated pooled swine serum in place of horse serum and a glucose concentration of 0·1%.

Buffered broth medium (*BBM*). BM was buffered with 0·2 M phosphate buffer, pH 7·5.

Chicken infusion broth (*CIB*). This was similar to that described by Jordan & Kulasegaram (1968) except that the infusion was autoclaved at 10 lb./in.^{2}, 114° C. for 30 min., and the pH indicator was phenol red.

Antisera

Rabbit sera. Two types of antisera were prepared: (1) two rabbits were immunized with BM and (2) four rabbits were immunized with a 24 hr. culture of *M. gallisepticum* that had been grown in BM, washed three times in 0·02 M phosphate buffered saline (PBS), pH 7·0, and adjusted to a standard optical density (Bradbury & Jordan, 1971*b*). The inoculation procedure was as described by Jordan & Kulasegaram (1968) but a multiple emulsion (Herbert, 1967) replaced the single emulsion adjuvant.

Turkey sera were obtained from natural infections with *M. gallisepticum*. All sera were stored at 0–4° C. and heated at 56° C. for 30 min. before use.

Preparation and standardization of M. gallisepticum *antigens*

These procedures have been described by Bradbury & Jordan (1971*b*). Inocula were 10% (v/v) of a 24 hr. broth culture unless otherwise stated, and cultures were incubated at 37° C.

Detection of adsorbed medium proteins

The antiserum raised in rabbits against BM was used to detect adsorbed protein by a slide agglutination or a gel diffusion test.

Slide agglutination (*SA*), *haemagglutination* (*HA*) *and gel diffusion* (*GD*) *tests*

These were all performed as described by Bradbury & Jordan (1971*b*).

Table 1. *The influence of pH of the medium and incubation time on the adsorption of medium proteins to* M. gallisepticum (*Expt.* 1)

Incubation time (hr.)	pH of medium	Reactions of antigens with antiserum to BM	
		SA	Gel precipitation
24	6·8	–	–
48	5·2	–	–
72	5·1	+	–
120	5·1	+ + +	+
168	5·1	+ + +	+

SA: +, Positive reaction in 2 min.; + +, positive reaction in 1 min.; + + +, strong positive reaction within 30 sec.

Table 2. *The effect of culture in buffered broth medium (BBM) compared with BM on the adsorption of medium proteins to* M. gallisepticum (*Expt.* 2)

Incubation time (hr.)	Antigens from BBM		Antigens from BM	
	pH of medium	SA with anti-serum to BM	pH of medium	SA with anti-serum to BM
48	6·9	–	5·1	+
168	6·3	–	5·2	+ +

Immunoelectrophoretic analysis (IEA)

This was carried out by the microtechnique of Scheidegger (1955) using LKB* equipment. One per cent agar in veronal buffer (I = 0·025, pH = 8·6) was used on the slides and the electrode vessels were filled with veronal buffer (I = 0·1, pH = 8·6).

RESULTS

The influence of pH on the adsorption of media proteins

Experiment 1

One-litre volumes of BM were inoculated with *M. gallisepticum* and, after 24, 48, 72, 120 and 168 hr. incubation, the pH was measured, the organisms harvested and standardized antigens were prepared. The antigens were tested for the presence of medium protein by SA and GD using rabbit antiserum to BM and the results are shown in Table 1.

The pH of the medium had fallen to 5·2 by 48 hr. and medium proteins were detectable by SA after 72 hr. incubation when the pH of the medium had been low for at least 24 hr. After 120 hr. incubation the agglutination reaction was more rapid, with larger floccules, and a single precipitin line was seen in gel diffusion.

* Stockholm, Sweden.

Table 3. *The influence of pH of the medium on the adsorption of medium proteins to non-viable* M. gallisepticum *organisms* (*Expt.* 3)

pH of medium	SA reaction of antigens with antiserum to BM
7·0	−
6·0	±
5·0	+
4·0	+ + +
3·0	+ + +

±, Doubtful positive reaction.

Experiment 2

To investigate further the influence of pH, *M. gallisepticum* was grown in BM and BBM, and after incubation periods of 48 and 168 hr. antigens were prepared and tested as above.

Table 2 shows that although the pH of the buffered medium did fall, at the time of harvest it was considerably higher than the unbuffered medium. Antigens prepared from the buffered medium showed no detectable medium constituents in contrast to those from BM.

Experiment 3

To examine the effect of pH on the adsorption of protein to dead organisms, antigen was prepared from BBM after 2 days incubation and then stored at 0–4° C. until the organisms were no longer viable. Medium proteins could not be detected by SA. Equal volumes (1 ml.) of antigen were then added to 25 ml. BM that had been adjusted to the following pH values: 7·0, 6·0, 5·0, 4·0, 3·0. After incubation for 24 hr. at 37° C. the organisms were harvested and prepared in the usual way as serological antigens. Table 3 gives the results of slide agglutination tests with these antigens and shows that acid pH caused adsorption of medium proteins to the dead organisms.

Identification of the adsorbed proteins

Experiment 4

(i) To determine which of the medium constituents are antigenic, serum from rabbits immunized with BM was examined by GD against BM and against each individual component of BM that was likely to be antigenic (i.e. swine serum, peptone, beef heart infusion, yeast extract). BM and swine serum were the only materials that gave a positive reaction (Plate 1 A). The precipitin lines developed by whole BM showed reactions of identity with those of swine serum indicating that swine serum proteins were the only constituents of this medium to elicit a detectable precipitin response.

(ii) To determine which of the antigenic constituents of BM attach to the *M. gallisepticum* organism, rabbits were immunized as described earlier with washed organisms grown in BM. The resulting sera were examined by GD tests against BM and the possible antigenic components of BM. Plate 1 B shows three

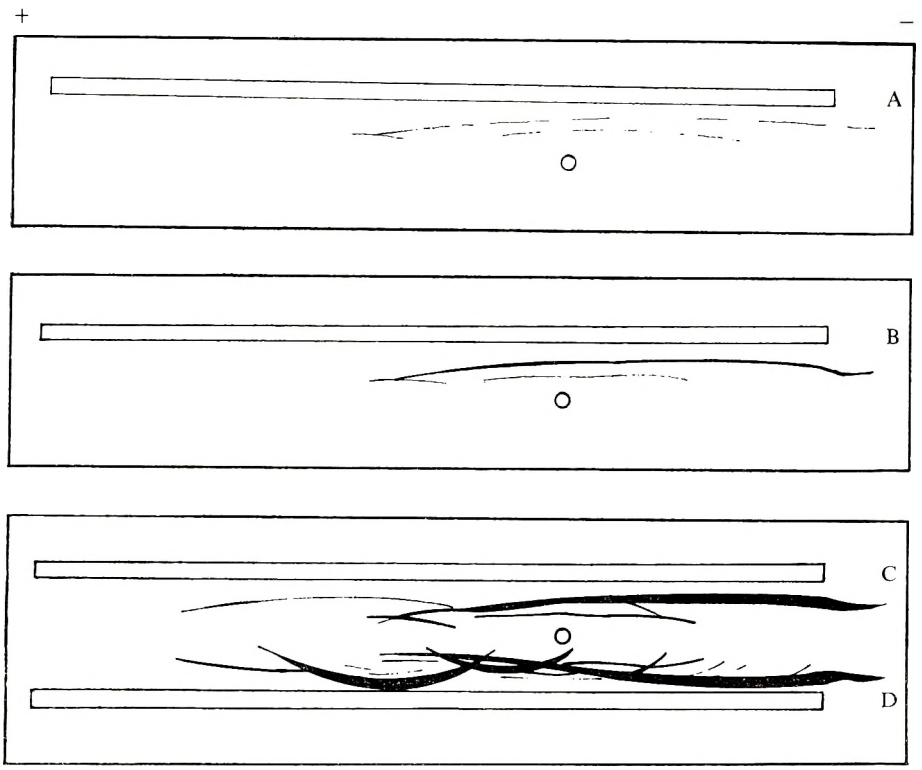

Fig. 1. Immunoelectrophoretic identification of swine serum proteins adsorbed to *M. gallisepticum*. The wells contained normal swine serum and troughs A, B and C contained rabbit antiserum to washed *M. gallisepticum* organisms. Trough D contained rabbit antiserum to whole swine serum.

precipitin bands, indicating that at least three medium constituents had resulted in antibody production; it further shows that the same three precipitin bands were developed against swine serum. Precipitating antibodies to the other medium constituents were not detected.

(iii) Immunoelectrophoresis was used to identify the swine serum proteins that attach to *M. gallisepticum*. Normal swine serum was separated by electrophoresis and two troughs were cut, one on either side of the path of separation. One trough was filled with a rabbit antiserum to washed organisms and the other with a rabbit antiserum to whole swine serum* for reference. The rabbits (six in number) had been immunized with washed organisms. Numbers 1–3 had received one immunizing course and the others, 4–6, had received two courses (Nutor, 1969).

Fig. 1A shows that sera from rabbits 1–4 had antibodies to three swine serum proteins. These were: (1) IgG, (2) a protein resembling IgM; (3) a protein of α-globulin mobility. Serum from rabbit 5 (Fig. 1B) showed antibody to the same three proteins but the precipitin arc in the α-globulin region was more readily seen. Serum from rabbit 6 (Fig. 1C) had antibody to two more proteins – one of prealbumin mobility and one of β-globulin mobility, possibly transferrin (Tormo, Chordi, Rodrigues-Burgos & Diaz, 1967).

* Nordic Pharmaceuticals.

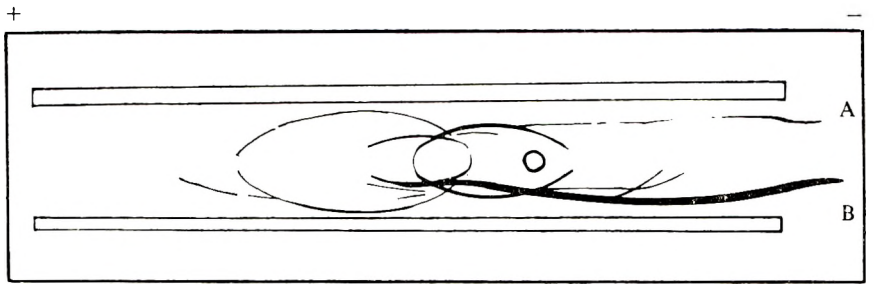

Fig. 2. Immunoelectrophoretic identification of swine serum proteins adsorbed to *M. gallisepticum*. The well contained normal swine serum and trough A contained rabbit anti-swine serum that had been incubated with *M. gallisepticum* organisms carrying adsorbed swine serum proteins. Trough B contained rabbit antiserum to whole swine serum.

Table 4. *The influence of pH of the medium and incubation time on the adsorption of chicken serum proteins to* M. gallisepticum (*Expt.* 5)

Incubation time (hr.)	pH of medium	SA reaction of antigens with antiserum to chicken serum proteins
18	6·0	−
48	5·0	±
168	5·0	+ + +

The slides were stained for lipoprotein with Oil Red O (Crowle, 1961) and the α-globulin showed a weak positive reaction.

(iv) To confirm the identity of the attached serum protein *M. gallisepticum* was cultured for 168 hr. in BM and standardized antigen was prepared. It was strongly agglutinated by antiserum to BM. A 1 ml. sample of this antigen was centrifuged at 3000*g* for 40 min., the supernatant was removed and 0·15 ml. rabbit anti-swine serum was added and left overnight at 0–4° C. so that any swine serum proteins on the antigen surface would absorb the corresponding antibody from the antiserum. After further centrifugation at 3000*g* for 40 min. the supernatant 'absorbed rabbit anti-swine serum' was used for analysis of electrophoresed normal swine serum. Unabsorbed antiserum was also used as a reference.

Fig. 2 shows that much antibody to IgG had been absorbed by the mycoplasmas. In addition antibody to two other proteins in the β–γ-globulin region had been absorbed. One of these corresponded to the IgM-like protein shown by all the sera in part (iii) above and the other corresponded to the probable transferrin shown by rabbit serum number 6 above. Antibody to pre-albumin had also been absorbed.

Experiment 5

To determine whether serum protein from a different species would adsorb to the organisms under similar conditions, *M. gallisepticum* was cultured in chicken infusion broth supplemented with chicken serum (CIB). Organisms were harvested, and standard antigens prepared after 18, 48 and 168 hr. incubation. The antigens

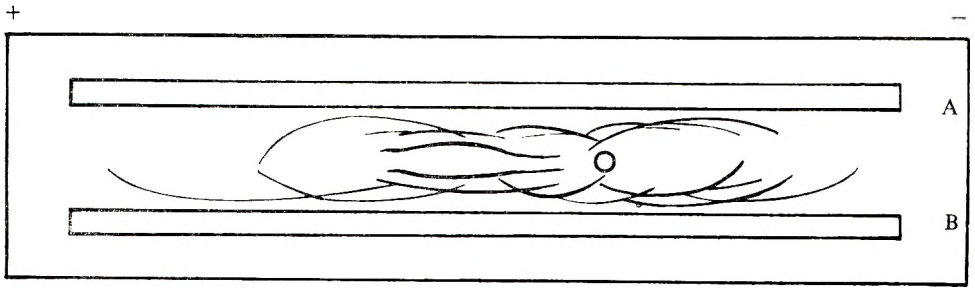

Fig. 3. Immunoelectrophoretic identification of chicken serum proteins adsorbed to *M. gallisepticum*. The well contained normal chicken serum and trough A contained rabbit anti-chicken serum that had been incubated with *M. gallisepticum* organisms carrying adsorbed chicken serum proteins. Trough B contained rabbit antiserum to whole chicken serum.

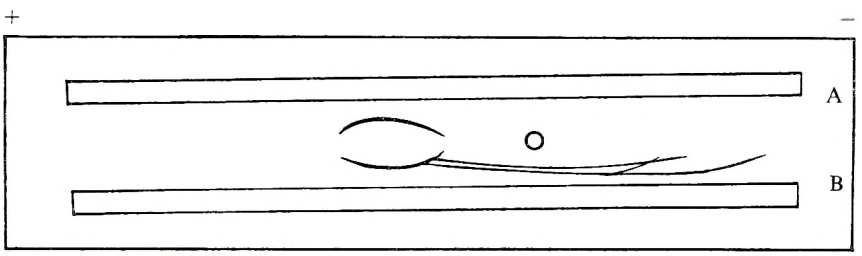

Fig. 4. Immunoelectrophoretic identification of chicken serum proteins adsorbed to *M. gallisepticum*. The well contained normal chicken serum and trough A contained rabbit antiserum to 4* chicken serum proteins and had been incubated with *M. gallisepticum* organisms carrying adsorbed chicken serum proteins. Trough B contained rabbit antiserum to 4* chicken serum proteins. * = IgG, IgM, transferrin and an α-globulin.

were then tested for the presence of adsorbed chicken serum proteins by slide agglutination with rabbit antiserum to chicken serum proteins.

Table 4 shows that chicken serum proteins were detected with increasing duration of incubation at low pH.

Experiment 6

To identify the attached chicken serum proteins an experiment similar to Expt. 4 part (iv) was carried out with antigen prepared after 168 hr. incubation in CIB. Rabbit anti-chicken serum was used instead of rabbit anti-swine serum for IEA and a rabbit serum with antibody to chicken IgG, IgM, transferrin and an α-globulin was also used.

Figs. 3 and 4 show that the proteins that were adsorbed probably correspond to some of those that were adsorbed from swine serum, i.e. IgG, IgM, transferrin and pre-albumin.

Table 5. *Reciprocal SA and HA titres of* M. gallisepticum *antigen after incubation in various media* (*Expt.* 7)

Incubation time (hr.)...	0	8	24	48	96	0	8	24	48	96
Serum	Antigen from medium A					Antigen from medium B				
SA 1	5	1	1	1	1	10	5	5	1	1
2	1	1	1	1	1	10	5	5	5	5
3	1	0	0	0	0	10	1	1	1	1
4	10	10	5	5	5	20	10	10	10	10
HA	32	16	8	4	2	16	8	4	4	4
	Antigen from medium C					Antigen from medium D				
SA 1	5	1	1	1	1	10	5	5	1	1
2	1	1	0	1	1	10	5	1	1	1
3	1	0	0	0	0	10	1	1	1	1
4	10	10	5	5	1	20	10	10	10	10
HA	64	64	32	32	32	32	32	64	64	32

Serum	Untreated antigen
SA 1	1
2	1
3	1
4	5
HA	64

Medium A = BM, pH 7·5; medium B = BM, pH 5·0; medium C = BM without swine serum, pH 7·5; medium D = BM without swine serum, pH 5·0.

The influence of adsorbed medium proteins on SA and HA reactivity of M. gallisepticum

Experiment 7

Antigen for this experiment was prepared from an *M. gallisepticum* culture after 24 hr. incubation in BM and medium proteins were not detectable by agglutination. Standardized antigen was inoculated in 1 ml. samples into 5 × 25 ml. volumes of each of the following media: (A) BM, pH 7·5; (B) BM, pH 5·0; (C) BM without swine serum, pH 7·5; (D) BM without swine serum, pH 5·0. The broths at pH 7·5 contained 0·4 M phosphate buffer so that pH change on incubation would be minimal. The other broths (B and D) were adjusted to pH 5·0 with lactic acid to simulate acid conditions produced by the mycoplasmas (Tourtellotte & Jacobs, 1960; Neimark & Pickett, 1960). Since 5·0 was the minimum pH reached by any *M. gallisepticum* culture in BM, it was not necessary to buffer these broths.

Standardized antigen was prepared immediately (0 hr.) from one sample of each type of broth. The other broths were incubated at 37° C. and antigen was prepared from one of each type after 8, 24, 48 and 96 hr. incubation.

All these antigens, together with a sample of the untreated antigen, were compared for sensitivity in SA tests with turkey sera from a naturally infected flock, and in HA tests.

The results, presented in Table 5, show that the antigen sensitivities in SA and

Table 6. *Reciprocal SA and HA titres of* M. gallisepticum *antigen* after *incubation in various media* (*Expt.* 8)

Incubation time (hr.)...	0	8	24	48	96	0	8	24	48	96
Serum	Antigen from medium A					Antigen from medium B				
SA 5	10	10	10	10	10	10	10	10	5	1
6	5	1	1	1	1	1	1	1	1	0
7	5	5	5	5	5	5	5	5	5	1
8	5	5	5	10	5	10	10	5	10	1
HA	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
	Antigen from medium C					Antigen from medium D				
SA 5	10	10	10	5	10	10	10	10	10	10
6	5	1	1	1	1	5	1	1	1	1
7	5	5	5	5	5	5	5	5	5	5
8	10	10	10	10	10	10	10	10	10	10
HA	2	4	2	< 2	2	2	2	2	< 2	2

Untreated antigen	
Serum	
SA 5	10
6	1
7	5
8	5
HA	4

Medium A = BM, pH 7·5; medium B = BM, pH 5·0; medium C = BM without swine serum, pH 7·5; medium D = BM without swine serum, pH 5·0.

HA tests are affected differently by the different media and by the duration of incubation.

For the slide agglutination test the sensitivity of the antigen was enhanced by lowered pH (B and D) although this declined on continued incubation. Swine serum appeared to be without effect.

For haemagglutination the sensitivity of the antigen was immediately reduced and progressively reduced on incubation in the presence of swine serum. The pH seemed to have little effect except that a slightly more rapid decline in titre was seen at pH 5·0 (medium B).

Experiment 8

This experiment was undertaken to confirm the earlier observations by Bradbury & Jordan (1971*b*) that antigens prepared from cultures after prolonged incubation at low pH reached a minimum of SA and HA sensitivity, and, therefore, unlike those from young cultures, would not be expected to be much affected by further treatment.

The experiment was therefore similar to Expt. 7 except that the antigen was from a 168 hr. culture that had been at low pH (5·0–5·1) for 120 hr. The results are shown in Table 6.

The SA sensitivity was not obviously altered by treatment with any of the four

media except for a slight decrease after 96 hr. in medium B (with swine serum, at pH 5·0). The HA titres were all low, but those antigens treated with media containing swine serum (A and B) showed consistently lower titres than antigens from media without serum (C and D).

DISCUSSION

Our results have shown that the pH of the culture medium influences the adsorption of medium proteins to the organisms. In Expt. 1 adsorbed proteins were detected after the pH had become low (5·1–5·2) for at least 24 hr, and the role played by low pH was further demonstrated in Expt. 2, in which prolonged incubation of the organisms in buffered broth did not result in adsorption while incubation for similar periods in unbuffered broth did. The pH of the medium was the only variable in Expt. 3 since the organisms used were non-viable. This experiment clearly confirmed that medium proteins attach in acid pH. They could not be detected after incubation of the organisms in broth of neutral pH.

Jordan & Kulasegaram (1968) found that, in broth medium, the proteins of the serum supplement were the only demonstrable precipitinogens. Expt. 4 confirmed this since rabbits that had been repeatedly inoculated with BM had detectable precipitins to swine serum but not to any of the other medium constituents.

Rabbits that had been inoculated with washed *M. gallisepticum* organisms also had precipitins to swine serum proteins but to no other medium constituents. It is relevant to note that these organisms had been in culture for only 24 hr. and yet had picked up sufficient serum protein to elicit specific antibody. It seems likely therefore that most *M. gallisepticum* antigen preparations will be carrying some serum proteins from the medium although they may be in amounts too small to be detectable even by agglutination. Other studies (unpublished observations) suggest that proteins may also become attached at other-than-acid pH, but again in amounts too low for detection by agglutination. It is not known whether the protein attaches by a physical bond or by an enzyme-mediated reaction, but the attachment appears to be irreversible and the proteins are not removed by extensive washing (Jordan & Kulasegaram, 1968). It is interesting in this context to note that Hirata & Stashak (1965) were able to adsorb bovine serum albumin onto formalin-treated erythrocytes at pH 5·0 and that the adsorbed protein was not removed by three washes in buffer at pH 7·2. Ovalbumin was found to adsorb at pH 4·0 but not at pH 5·0, which indicates that different proteins may require different pH values for adsorption. There was no likelihood of enzyme involvement in these reactions.

Immunoelectrophoretic analysis of the rabbit antisera to washed *M. gallisepticum* organisms revealed that only certain swine serum proteins including IgG and IgM had elicited an antibody response. It is possible that all the serum proteins had become non-selectively attached to the organism and that the rabbits made a better immune response to some than others; however, this in unlikely in view of the absence of antibody to albumin, which is a powerful antigen and accounts for approximately one-third of the total protein of swine serum. A more likely

explanation is that certain swine serum proteins were adsorbed preferentially. In similar experiments Hamburger *et al.* (1963) identified horse IgG as the major contaminating protein on washed HeLa H cells that had been grown in medium containing horse serum, and in 1967 Beernink & Steward, studying the attachment of guinea-pig serum proteins to *Escherichia coli*, were able to demonstrate the presence of seven globulins on the organism. Of these, IgG, IgM and a β-globulin were most consistently found.

The attachment of serum proteins to the *M. gallisepticum* organisms is not confined to swine serum. In Expt. 5 chicken serum proteins were detected in increasing amounts with incubation and accompanying fall in pH. As with swine serum, the proteins were adsorbed selectively. It is of some significance that these proteins include immunoglobulins which could perhaps play a contributory role to the false positive reactions that are frequently encountered in serological tests involving *M. gallisepticum* and avian sera (Bradbury & Jordan, 1971*c*).

Asmar (1965), in a study of chicken and turkey agglutinating antibodies to *M. gallisepticum*, found that certain proteins in non-agglutinating sera were adsorbed to the organism. The proteins were detected after incubation of the sera with antigen in buffer at pH 8·0 and the bound chicken serum proteins were characterized by Asmar as gamma, beta 2 M, beta 1 C and alpha 1 globulins. The first three may well correspond to the IgG, IgM and transferrin picked up by *M. gallisepticum* in culture in our studies. Asmar's findings support the suggestion that serum proteins adsorb to the organism at other than acid pH.

Expt. 7 revealed an association between the presence of swine serum proteins and the HA properties of the *M. gallisepticum* organism, for when an antigen was prepared from a 24 hr. culture in broth with swine serum and then further incubated in fresh broth of similar constitution, the HA titre was progressively diminished. This apparent blocking effect was not seen when such an antigen was incubated in broth without swine serum. The adsorption of serum protein may thus explain why *M. gallisepticum* organisms in routine culture lose their HA titre on continued incubation. In contrast, the SA sensitivity of the antigen in this experiment was not apparently influenced by the presence or absence of swine serum but the results did confirm an earlier observation by Bradbury & Jordan (1971*b*) that brief incubation of the organisms at low pH actually enhances their SA sensitivity although it declines on further incubation. This aspect is under further investigation in view of the wide application of the SA test for the detection of small amounts of antibody in poultry flocks.

When an antigen was prepared from a 168 hr. culture and then treated as in the previous experiment, the sensitivity in SA and HA tests was not noticeably affected by either the presence of swine serum or the pH. This was probably because the sensitivity had already declined following the long (168 hr.) culture period (Bradbury & Jordan, 1971*b*).

Since these studies have shown that the HA reactivity of *M. gallisepticum* is reduced by prolonged incubation in serum-supplemented medium, it would be of interest to know whether other properties of the organism are similarly affected.

We wish to thank Mrs C. A. Barratt for technical assistance and the Agricultural Research Council for financial support.

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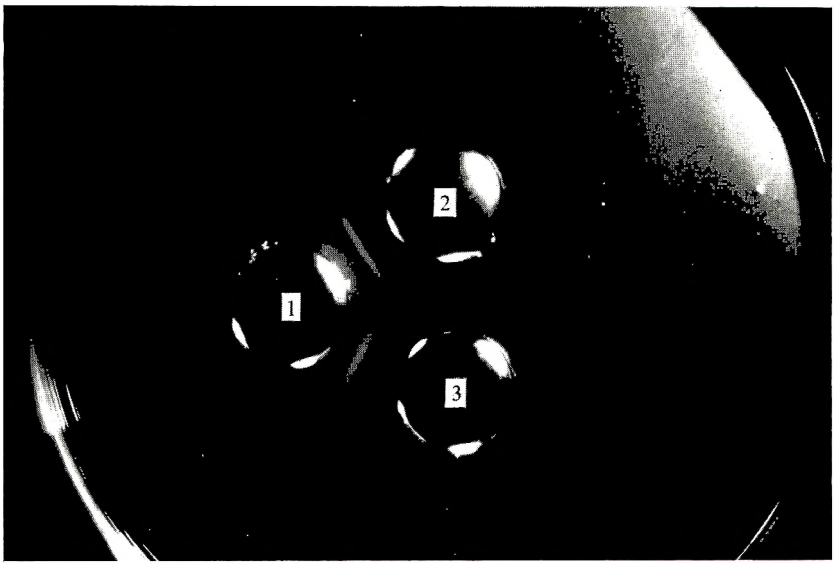
EXPLANATION OF PLATE

A. Precipitin reactions of rabbit antiserum to broth medium (1) with broth medium (2) and swine serum (3).

B. Precipitin reactions of rabbit antiserum to washed *M. gallisepticum* organisms (1) with broth medium (2) and swine serum (3).



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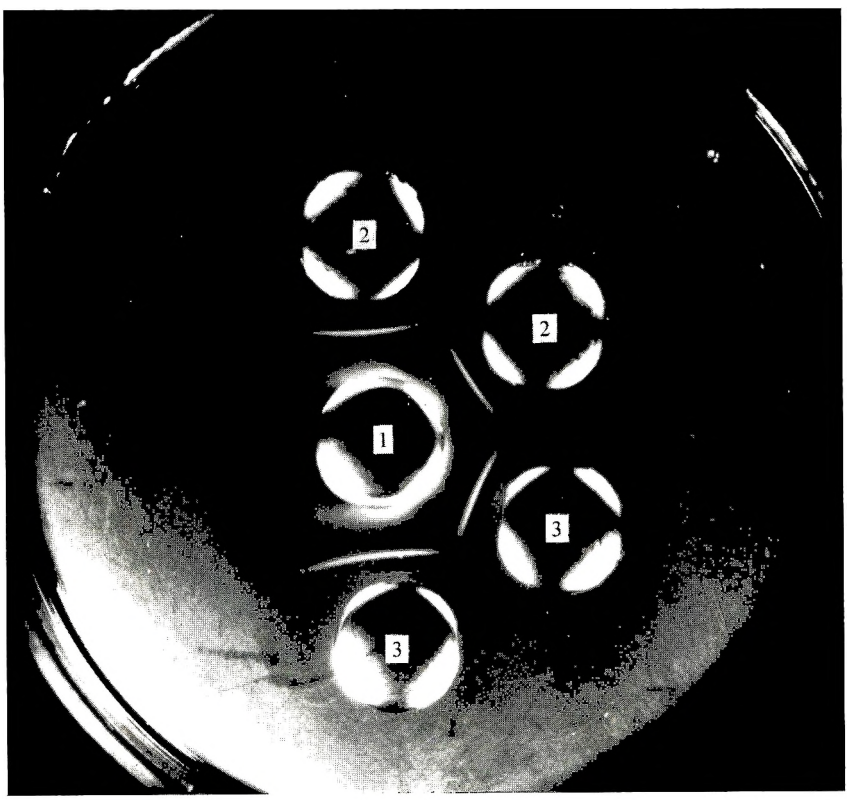

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The control by ventilation of airborne bacterial transfer between hospital patients, and its assessment by means of a particle tracer

I. An airborne-particle tracer for cross-infection studies

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SUMMARY

A simple and convenient particle tracer for studies of the effectiveness of isolation units and other places in limiting the airborne transfer of bacteria is described. Particles of potassium iodide 7–8 μm. diameter are generated by spraying from solution and collected on membrane filters. The particles can be identified by development with 0·1 % acid palladium chloride solution, when dark brown spots approximately 100 μm. in diameter are produced.

INTRODUCTION

Although the relative importance of the airborne and contact routes for the transfer of infection is still uncertain, isolation units are constructed with elaborate and expensive ventilation systems designed to eliminate the transfer of airborne micro-organisms between patients. It is, moreover, common experience that many such installations do not function in accordance with their design specification.

It is possible (Lidwell, 1972), by making some simplifying assumptions, to estimate the capability for air isolation of a particular design. In order to check the actual performance of a unit under working conditions some form of airborne tracer must be used. Identifiable strains of micro-organisms carried by the patients may be used for this purpose (e.g. Williams & Harding, 1969), but although such a method is closer to the real issue than is the use of gas or inert particulate tracers it has several limitations. The dispersing sources are not under control, and the relatively small numbers of identifiable airborne particles dispersed means that the method is rather insensitive and that observation must be carried out over a prolonged period.

Nitrous oxide has been used as a gaseous tracer (e.g. Lidwell & Williams, 1960; Baird, 1969), but the instruments available for measuring it are of limited sensitivity, and determination to an accuracy of 10 % requires concentrations of the order of $1/10^4$ in air. Any higher sensitivity would often lead to difficulties of interpretation in a hospital because of the widespread medical use of this gas. Much greater sensitivities can be obtained by the use of ion-capture detectors to estimate low concentrations of some halocarbons and similar compounds (N. Foord

& O. M. Lidwell, to be published). Estimations to within 10 % can be obtained at concentrations as low as $1/10^9$ with some compounds, and the use of a column for chromatographic separation also allows more than one tracer gas to be estimated at one time, thus allowing simultaneous study of transfer from several source positions.

Airborne micro-organisms are however necessarily particulate and the particles responsible for transferring bacteria through the air are generally large enough to have a settling velocity in air (Noble, Lidwell & Kingston, 1963) of the order of 0·3 m./min. This may lead to substantial quantitative and perhaps qualitative differences in the pattern of dispersion of these particulates compared with the dispersion of a gaseous tracer. In addition, if there is any recirculation in the system, gases will not be retained in the filters, and no estimate of particulate dispersion can then be made by gas tracer studies. A satisfactory particle tracer would therefore be a useful tool for assessing the performance of an isolation system.

Fluorescent particles have been used as particulate tracers and an elegant method has been devised by Goldberg (1968) for distinguishing the tracer particles from other kinds of airborne particles that may produce spurious signals in the detecting equipment. However, although this system is very sensitive and has the advantage of giving essentially instantaneous readings, the equipment is elaborate and does not readily lend itself to simultaneous estimates of the concentration of particles at many sites at one time. Bacterial tracers have been widely used in some situations (e.g. Lidwell & Towers, 1969), but there are [illegible] to the widespread dissemination of any micro-organism, no matter how apparently innocuous, in a working hospital.

The ideal particulate tracer would be imperceptible, innocuous, easy to produce and disperse in a range of particle sizes, and such that individual particles are easily recognized and counted after collection.

DEVELOPMENT OF A METHOD

Particles of a controlled size are perhaps most easily produced in the numbers required by spraying a solution or dispersion of the selected substance in a volatile solvent. Simple air-driven sprays, or humidifying devices in which the liquid is thrown from a revolving disk against a set of stationary teeth, produce droplets with a wide size range and the particulate cloud is therefore inhomogeneous. The sizes of the particles carrying airborne micro-organisms also vary over a wide range but the degree of inhomogeneity is not necessarily the same as that of the sprayed droplets and variations in size make the detection and accurate counting of the particles more difficult. Spinning disk sprays, driven either by air or electrically and capable of producing a homogeneous cloud of droplets down to diameters of 40 μm. or less, are commercially available.

Our first experiments were carried out by spraying aqueous solutions of sodium chloride. When the resulting particles were collected by impingement or settling on Petri dishes filled with 0·5 % agar (Oxoid Ionagar) containing silver nitrate the

Table 1. *Spot sizes obtained by collecting sodium chloride particles on 0·5 % ion agar containing silver nitrate, spot diameters in micrometres*

Conc. NaCl sprayed (%)	Particle diameter (μm.)	Conc. $AgNO_3$ (%)				
		1	0·5	0·1	0·01	0·001
8·3	10·3	—	—	135	—	—
2·5	7·6	46	58	98	180*	250†
0·3	3·7	—	—	44	—	—

* These spots were rather faint.

† These spots were very faint. Spots could not be detected if the silver nitrate concentration was below 0·001 %.

resulting approximately hemispherically shaped deposits of silver chloride could be converted by exposure to light, preferably long-wave ultra-violet, or by treatment with very dilute photographic developer, into well-defined nearly black disks. Table 1 shows the diameter of the resulting spots for different concentrations of silver nitrate and three sizes of sodium chloride particles. Although samples of air collected outside the laboratory contained fewer than one sodium chloride particle apparently 5 μm. or larger, per 50 l. the numbers found inside the building were 20–30 times greater, so that sodium chloride is not a suitable tracer for use within occupied buildings, nor can silver nitrate agar be used for the detection of other halides. Particles of water-soluble iodides can however be detected by other methods, in particular the reaction with palladous chloride, without interference from chloride.

Potassium iodide as a tracer particle

Potassium iodide particles, produced by spraying a solution of the salt, are collected by drawing the air to be sampled through a membrane filter. The particles are made visible by treating the membrane with a solution of palladous chloride. If prepared under suitable conditions the membranes when dried are stable and can be stored for long periods. The best spots were obtained by laying the membranes carefully onto the surface of the palladous chloride solution. The membrane then wets through rapidly. The spots of dark brown insoluble palladous iodide form almost instantaneously and the membranes can be lifted off and placed on absorbent paper to dry. A rinse in water before drying improves contrast by removing the excess palladous chloride and a large proportion of any dust particles which have been collected. The diffusion conditions on the surface of a membrane filter are not so well defined as on an agar plate and the resulting spot size is variable and not so simply related to the size of the particle and the concentration of the reagent as it is with the sodium chloride particles described in Table 1. Table 2 shows the average spot sizes obtained with potassium iodide particles of 7 μm. diameter, prepared by spraying a 5·6 % aqueous solution of the salt from a 25 mm. diameter spinning disk rotating at approximately 1000 rev./sec. As the size of the potassium iodide particle was reduced the diameter of the developed spot became only slightly smaller but its colour became fainter and its edges less distinct and spots arising from particles smaller than 3·5 μm. were very difficult to identify.

Table 2. *Spot sizes obtained by collecting potassium iodide particles (7 μm. diameter) on a membrane filter* and developing with palladous chloride solution, spot diameters in micrometres*

Conc. $PdCl_2$ (%)			
1	0·5	0·2	0·1
60	70	90	95

* Millipore SSWP 02500.

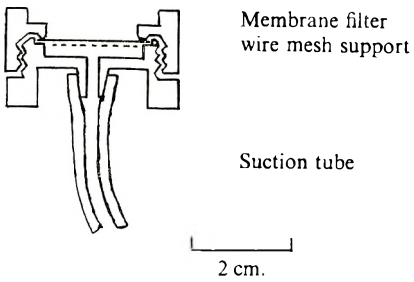
The grade of membrane filter used had little or no effect on the spot size but the spots were better defined the smoother the surface of the membrane.

The palladous chloride must be dissolved in an acid solution – N/10 hydrochloric acid is convenient – and should be filtered if cloudy. It keeps indefinitely, although after much use it may be necessary to filter it. The best concentration to use depends on the particle size of the potassium iodide. Denser colour but smaller spots are obtained with the stronger solution, so that smaller particles are visible after development. We have been interested in particles of 7–8 μm. diameter, with a settling velocity in air of approximately 0·3 m./min., corresponding to the median diameter commonly observed for airborne bacteria-carrying particles in the hospital situation. For particles of this size a 0·1 % solution gives good spots nearly 100 μm. in diameter, which can be easily seen and counted with a low-power (× 10) binocular plate microscope. At this power the field of view can be more than 20 mm. diameter, which is enough to cover the whole of the collecting surface of a 25 mm. membrane filter. Plates 1 (*a*) and 1 (*b*) show the spots as seen under a counting grid. Those in Plate 1 (*a*) are derived from particles of uniform size, approximately 8 μm. in diameter, obtained by spraying a 3·5 % solution of potassium iodide in 80 % ethyl alcohol from a spinning disk similar to that referred to above. Alcoholic solutions are usually preferable since it is then easier to ensure uniform wetting of the disk. The spots are relatively uniform in size and easy to identify. Plate 1 (*b*) shows spots derived from the particles produced by an air-driven baffled spray with approximately the same median diameter. The wide range of sizes leads to great variability in the visibility of the spots and it is difficult to establish a cut-off point for counting.

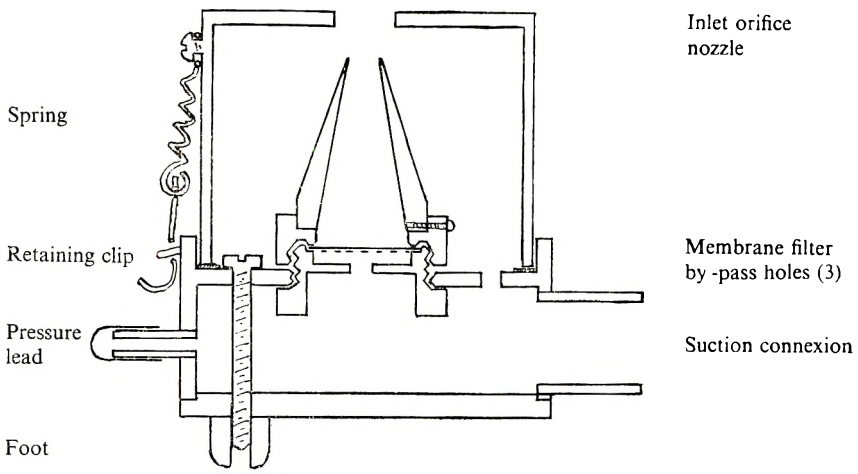
Collection of particles

The simplest method is to use a small plastic membrane filter holder, e.g. the Gelman easy pressure filter holder with the cone on the inlet side machined away (Fig. 1 (*a*)). With simple filter holders of this kind the rate of sampling is limited by the resistance of the filter. This varies with pore size and with different makes and batches of membranes. At 20 cm. water pressure the flow through a 25 mm. diameter filter in its mount varies from not much more than 1 l./min. for a pore size of 0·8 μm. to nearly 4 l./min. at a pore size of 5 μm. We have generally used a pore of size 3 μm. (Millipore Filter Corporation, Bedford, Mass., U.S.A., ref. SSWP 02500) which gives a flow rate around 3 l./min. at the above suction pressure.

Increased sampling rates can be obtained by the use of a centripetal sampler of the kind described by Hounam & Sherwood (1965). A single-stage version only is



(*a*)



(*b*)

Fig. 1. (*a*) Section of plastic filter holder (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) adapted for air sampling. (*b*) Centripetal sampler: orifice diameter, 1·25 cm. ($\frac{1}{2}$ in.); nozzle diameter, 0·62 cm. ($\frac{1}{4}$ in.); orifice nozzle spacing, 0·62 cm. ($\frac{1}{4}$ in.); outer cone angle, 20° + 20°; by-pass holes (3), 0·41 mm. (0·16 in.) diameter. Cone and cap of brass, body of aluminium alloy, plastic filter holder the same as (*a*).

required and a convenient form of the device is shown in Fig. 1(*b*). With the dimensions given, a sampling rate of 100 l./min. is reached with a suction pressure of 20 cm. of water but only 3 l./min. passes through the filter. Inertia, however, carries a high proportion of particles above a certain size in the sampled air into the axial flow from which they are collected by the filter. Langmead & O'Connor (1969) have given a theoretical analysis of this type of sampling system. For the geometry illustrated in Fig. 1(*b*) near 100% collection would be achieved for particles of potassium iodide (density 3·1) above 5·5 μm. in diameter at a sampling rate of 100 l./min., and 50% collection of particles 3·7 μm. in diameter. For particles of 7 μm. in diameter 50% collection efficiency is reached at a flow of 28 l./min. and near 100% at flows above 63 l./min. Experimental observations largely confirmed these calculations. Efficiencies of 80–100% were obtained with particles approximately 7 μm. in diameter at flows of 60 l./min. or more. Below this flow rate the

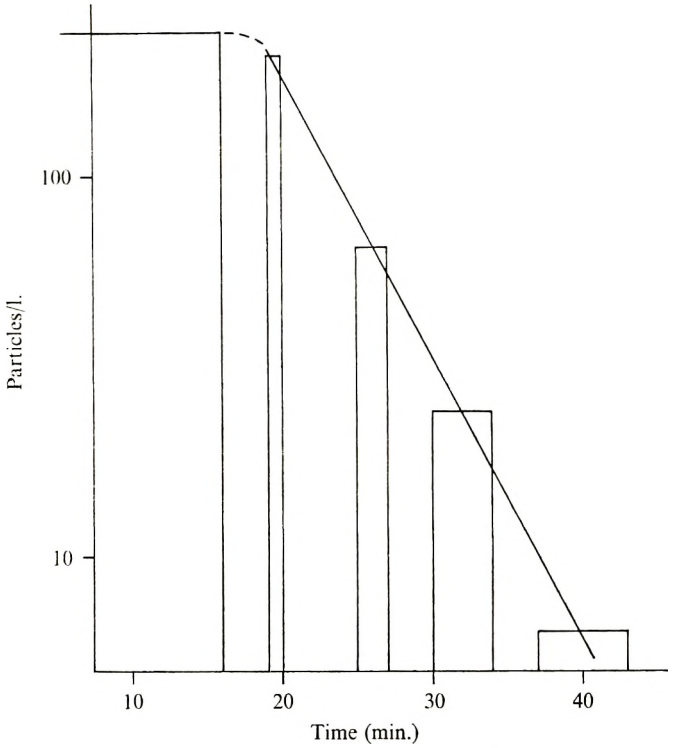


Fig. 2. Die-away rate of 7–8 μm. potassium iodide particles in patient room. The height of each column indicates the average concentration of particles during the sampling period. Spraying was discontinued at the end of the 16th minute.

efficiency appeared to fall off rather more rapidly than calculated to around 20 % at a flow rate of 30 l./min. Failure to collect very small particles is advantageous in practice. Potassium iodide particles below 3·5 μm. are in any case not detectable, while a large fraction of the material which causes blackening of the filter when sampling urban air is found in the smallest size ranges.

Particles of potassium iodide may also be collected by direct sedimentation on the surface of a membrane filter either dry or moistened with palladium chloride solution. Since the rate of deposition is normally very low, approximately equivalent to 0·1 l./min. on a 20 mm. diameter circle for particles of 7–8 μm. diameter with a settling velocity of 0·3 m./min., this is not usually a convenient method, but it does provide a direct method of estimating the settling velocity of the particles generated by any dispersing device.

Determination of the particle settling velocity

A 3·5 % solution of potassium iodide in 80 % ethyl alcohol was sprayed from a spinning disk atomizer into a room 3 m. × 5 m. × 3 m. high. The room was ventilated with approximately 200 m.^{3}/hr. of fresh air. After spraying had been in progress for 5 min., samples were taken at 3 l./min. through membrane filters at two positions in the room for the next 11 min. while at the same time two filters were exposed for settling. The number of particles collected on the two suction samples were esti-

mated to be 6700 and 9150 respectively. The numbers collected on the settle samples were 282 and 295. The area of the 20 mm. circle counted is π cm.^{2}. Hence settling velocity (s) is given by

$$s = \frac{282+295}{2\pi} \times \frac{2 \times 3000}{6700+9150} \text{ cm./min.}$$

$$= \frac{557}{2\pi} \times \frac{60}{15{,}850} \text{ m./min.}$$

$$= 0{\cdot}35 \text{ m./min.}$$

After the end of the 16th min. spraying was discontinued and samples taken at 3 l./min. at three positions in the room over the periods 19–20th, 25–27th, 30–34th and 37th–43rd min. The numbers of particles obtained in these samples are shown graphically in Fig. 2, from which the estimated die-away rate is 10·3/hr. Since the room has a volume of 45 m.^{3} the ventilation of 200 m.^{3}/hr. is equivalent to an air change rate of 4·5/hr. The extent by which the die-away rate exceeds this figure can be attributed to particle settling, i.e. a rate equivalent to 5·8 air changes/hr. Since the room was 3 m. high this corresponds to a settling rate (s) of $3 \times 5{\cdot}8$ m./hr. or 0·29 m./min.

The difference between this value and that of 0·35 m./min. estimated from the ratio of the volumetric concentration of particles to the rate of settling onto a horizontal surface is certainly within the experimental errors of this determination. Together the observations confirm that the potassium iodide particles produced had the desired settling rate of approximately 0·3 m./min. The room used for this experiment was one of those forming the burns unit in which a systematic study of particle transport from room to room has been carried out using the particle tracer method described (Hambraeus & Sanderson, 1972).

Discoloration of floors by settled particles

Under some circumstances particles of potassium iodide which have settled on floors or other surfaces may oxidize and give rise to yellow iodine staining. This is not troublesome unless there is an unusually large amount of material deposited in any one place, as may happen from settling of large particles or droplets in the immediate vicinity of an imperfectly baffled humidifier or air-driven spray. The minimum density of deposit which we have found to produce a just perceptible stain on white- or light-coloured polyvinyl floor tile when left undisturbed is about 3 mg./m.^{2}. The use of cleaning agents containing hypochlorite or other powerful oxidizing agents is, of course, liable to accentuate this effect.

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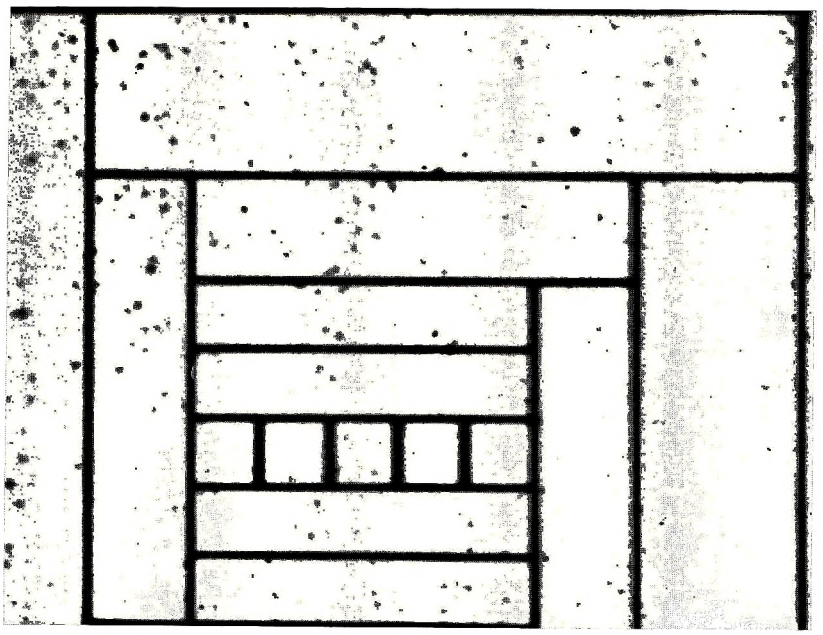
EXPLANATION OF PLATE

(*a*) Spots obtained on developing potassium iodide particles of nearly uniform size (*ca.* 8 μm.) produced by spraying a 3·5 % solution in 80 % ethyl alcohol from a spinning disk atomizer. The small squares in the centre of the counting grid shown over the developed filter are 0·8 × 0·8 mm. and each enclosure 0·2 % of the 20 mm. diameter sampling area on the 25 mm. diameter filter. The shadows alongside some of the grid lines arise from the combination of oblique two-sided illumination and imperfect contact between the graticule and the surface of the filter. (*b*) Similar to (*a*) except that the particles were generated by a baffled air spray, a simple scent spray head, using a 25 % aqueous solution of potassium iodide and an air pressure of 1·2 kg./cm.^{2}. There is a wide variation in the size of the particles produced.



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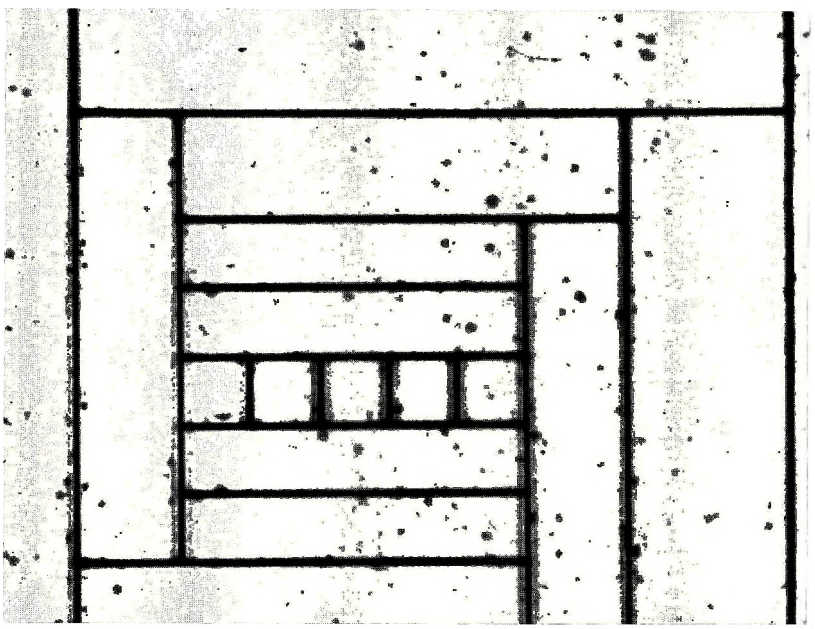

(*b*)

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The control by ventilation of airborne bacterial transfer between hospital patients, and its assessment by means of a particle tracer

II. Ventilation in subdivided isolation units

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SUMMARY

Values are deduced for the efficiency of isolation against airborne particulates, e.g. micro-organisms, of a variety of ventilation systems. The calculated values show reasonable correspondence with the limited experimental data available. Much better control and indication of the air flow is necessary if high degrees of isolation are required.

INTRODUCTION

In an isolation unit ventilation may serve any or all of three purposes, to maintain a comfortable environment, to remove smells, to reduce the possibilities of airborne transport of micro-organisms from one patient to another. The criteria for the first two purposes are well known and not difficult to meet. The third, however, is less clear. Epidemiological evidence as to the relevance of airborne infection is generally lacking so that a precise specification cannot be given. It seems likely, however, that if any clinical benefit is to be obtained it will only be gained by reducing the extent of airborne transport by several orders of magnitude. There are very few data on the physical effectiveness of different ventilation systems, but some deductions can be made from a theoretical analysis and these then compared with the scanty material available.

As a simplified model we may consider a number of rooms opening off a communications area (Fig. 1).

EXCHANGE OF AIR BETWEEN ROOMS

In calculating the transport of particles from the air exchange between the parts of a system, it is convenient to assume that turbulent air mixing within each room is sufficient to produce a substantially uniform concentration of particles within it. This is a satisfactory approximation so long as the spaces involved are not too large or unusually shaped.

In assessing the performance of any system it is more realistic to compare it with the situation in which all the patients are housed in one room having the same

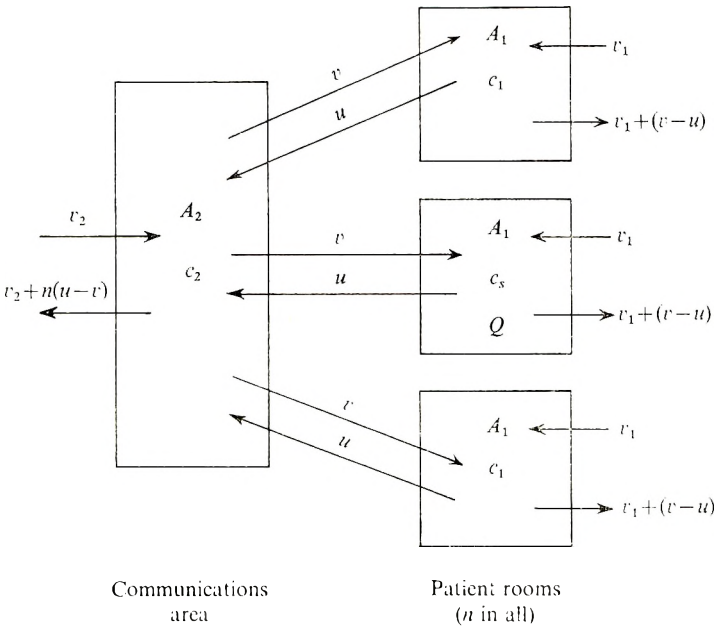

Fig. 1. Schematic diagram of the system analysed. The arrows show the directions of the air movements between the n patient rooms and the communications area together with the air supply and consequent exhaust. The symbols u and v, with suffixes as appropriate, indicate the volumetric rates of air movement, for example in m.^{3}/hr. Symbols c_1, c_2 denote the equilibrium particle concentrations arising from a source, Q particles per unit time (e.g. per hr.), in one patient room where the equilibrium concentration is c_s. A_1 and A_2 are the floor areas (more properly the aggregate areas of the horizontal surfaces on which particle sedimentation takes place) of the rooms.

total ventilation and floor area than just to compare the particle concentration in a room housing a source with that in the other patient rooms. The ratio of these concentrations, α in equation (5) below, which is all that is available for measurement by observations made within the system itself, gives an unduly favourable estimate of the efficiency of the isolation since the particle concentration in the source room is increased when the source is contained in a small room.

If, then, there is a source generating Q units of particle contamination/hr. in one of the rooms of the system shown in Fig. 1 the following equations will define the equilibrium state, neglecting the effect of particle settling

$$c_s(v_1+v-u)+c_s u = vc_2+Q, \tag{1}$$

$$c_1(v_1+v-u)+c_1 u = vc_2, \tag{2}$$

$$c_2(v_2+n(u-v))+c_2 nv = uc_s+c_1(n-1)u, \tag{3}$$

where the symbols have the meaning shown in Fig. 1.

In addition if the system were replaced by a single space with a floor area and ventilation supply equal to the sum of those of the individual rooms and the communications area then the equilibrium state would be defined by

$$c_m(v_2+nv_1) = Q. \tag{4}$$

This value of the particle concentration, c_m, is the value to which c_s, c_1 and c_2 all tend as u and v both become very large but $v \sim u$ remains small.

From equations (1)–(4) it is easy to deduce that

$$c_s/c_1 = \alpha = 1 + (v_1 v_2 + v_2 v + nuv_1)/uv, \tag{5}$$

$$c_m/c_1 = \beta = (v + v_1)(v_1 v_2 + v_2 v + nuv_1)/uv(v_2 + nv_1). \tag{6}$$

If both u and v are small relative to v_1 and v_2 then

$$\alpha = c_s/c_1 \doteq v_1 v_2/uv, \tag{5A}$$

$$\beta = c_m/c_1 \doteq v_1 v_1 v_2/uv(v_2 + nv_1), \tag{6A}$$

and

$$\alpha/\beta = c_s/c_m = n + v_2/v_1. \tag{7}$$

In addition

$$c_s/c_2 = \alpha' = 1 + (v_1 v_2 + v_2 v + \overline{n-1}\, uv_1)/u(v_1 + v), \tag{8}$$

$$c_2/c_1 = \alpha'' = 1 + v_1/v, \tag{9}$$

or if both u and v are small as before

$$\alpha' = c_s/c_2 \doteq v_2/u, \tag{8A}$$

$$\alpha'' = c_2/c_1 \doteq v_1/v. \tag{9A}$$

In all the above equations u and v, the volumes of air passing between the individual rooms and the communications area, include air movements due to imbalance in the air supplied to or extracted from the rooms, air exchange across the doorways or other openings as a result of thermal differences and air exchanged across doorways when the doors are opened and shut and when people pass through them.

The effects of particle size

In addition to the effects of ventilation, the particles carrying the micro-organisms will be settling under the influence of gravity. The effect of this on the dispersal and air-concentration of the particles is equivalent, under conditions of complete turbulent air mixing within the individual rooms, to adding to the clean air input into any room a quantity As, where A is the floor area of the room concerned and s is the effective settling velocity of the particles. For bacteria-carrying particles the value of s is usually close to 0·3 m./min. (Noble, Lidwell & Kingston, 1963).

Transport through airlocks

In order to apply the results of the preceding section to systems which include airlocks, with doors to both the patient room and the communications area, it is necessary to obtain some estimate of the effective transfer of air through them when a person enters or leaves the room, i.e. of the rate of direct transfer of air from the room to the corridor, or in the reverse direction, that would transfer the same amount of airborne contamination as that effected by passage through the airlock. These estimates can then be introduced into equations 5–9 as the appropri-

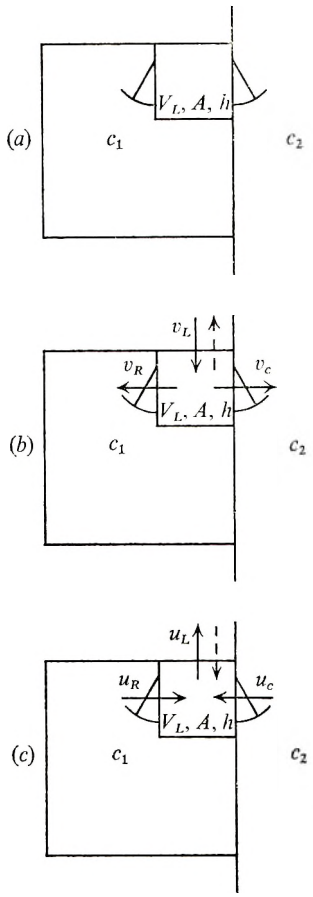

Fig. 2. Airlocks. Symbols c_1, c_s and c_2, as in Fig. 1, denote the equilibrium particle concentrations in the n patient rooms and in the communications area. V_L is the volume of an airlock, A its floor area and h its internal ceiling height. (*a*) Unventilated airlock. (*b*) Input ventilation at v_L of which v_R passes into the patient room and v_c into the communications area. (*c*) Extract ventilation at u_L of which u_R is drawn from the patient room and u_c from the communication area.

ate values of u and v. Three situations are illustrated in Fig. 2: (*a*) an unventilated lock; (*b*) a lock with clean air input; (*c*) a lock with extract.

The direction of air flow around the door edges with a balanced ventilation system and an unventilated lock are indeterminate. If there is no flow in either direction when the doors are shut then $u = v = mw^2/V_L$, when there are both m entries into the room and m exits from the room through the airlock in unit time, w is the volume of air exchanged across a door opening each time a door is opened and shut and V_L is the cubic volume of the lock. Loss by sedimentation within the lock is small, a dwell time of about 6 min. would be necessary to halve the concentration of particles within the lock even if it were no more than 2·5 m. high, given the assumed settling velocity of 0·3 m./min. A consequence of this slow die-away, however, is that particles already introduced into the lock may be transferred by a subsequent passage through. For an assumed rate of five passages/

hr. in each direction, one in either direction every 6 min., this will approximately treble the particle transfer,

$$u = v = mw^2/V_L\,(1+\tfrac{1}{2}+\tfrac{1}{4}+\ldots+\tfrac{1}{2}+\tfrac{1}{4}+\ldots) = 3mw^2/V_L.^*$$

In practice there will almost certainly be a flow in one direction or the other, due to residual imbalance in the ventilating system so that either u or v will be many times larger than this figure.

For locks with clean air input greater than any extract, the air in the lock will be clean in the absence of any passage through. Even with an input of 200 m.^{3}/hr. into a lock of volume 10 m.^{3}, 80 % of any contamination introduced on entering the lock will remain after a dwell time of 0·01 hr.; but this is of no significance, since any contamination introduced by opening doors will be distributed to the two sides of the airlock in proportion to the air flows. The effect of this is identical in whichever direction a person passes through the lock, so that $u = 2\,mwv_C/v_L$ and $v = 2mwv_R/v_L$. If there is no extract and $v_C = v_R = \frac{1}{2}v_L$ then $u = v = mw$. Loss by settling during the time taken to flush the contamination out of the lock will however reduce the effective particle transfer. The proportion so lost by settling is given by $60sA/(60sA + v_L)$. If the air input is 100 m.^{3}/hr., the settling area within the lock is 4 m.^{2} and s is taken as 0·3 m./min. then the proportion so lost $= 72/(72+100) = 0{\cdot}42$. If $v_C = v_R = \frac{1}{2}v_L$ then as far as the transport of particles through the airlock is concerned effectively $u = v = (1-0{\cdot}42)mw = 0{\cdot}58mw$. The net air input to the lock must be regarded as additional ventilation air to the system, divided according to the proportionate air flows in the two directions (i.e. v_R is to be included in v_1 and nv_C in v_2). Any contamination produced by activities within the airlock, e.g. putting on or taking off protective clothing etc., will be carried both into the room and into the communications area in proportion to the air flows, subject to losses from sedimentation similar to those described above.

The air within an airlock with extract in excess of any input is always contaminated from both sides in proportion to the relative input flows from both sides. Dwell time in the lock is therefore again irrelevant, since it is not entry into the lock that introduces the airborne particles. On entering or leaving the lock, particles are transferred across the door concerned, hence $u = 2mwu_R/u_L$ and $v = 2mwu_C/u_L$. If there is no input and $u_R = u_C = \frac{1}{2}u_L$ then $u = v = mw$. The continuous loss by sedimentation within the airlock will reduce this to a similar extent to that given above for the positive input situation substituting u_L for v_L. Contamination produced by activities within the lock itself will be transferred in the direction of subsequent passage. With little effect from any dwell time of less than 2–3 min., the fraction will approximate to w/V_L, usually substantially less than the amount transferred from a positively ventilated lock.

Further reduction in the transfer through an airlock can be obtained by a high

* This assumes that w/V_L is sufficiently small for the reduction in concentration of particles left in the airlock each time a volume w is lost on opening a door to be negligible. If w/V_L is as large as 1/10 when the other conditions are as assumed above then the values of u and v will be reduced to approximately $2{\cdot}2mw^2/V_L$.

rate of near balanced ventilation to the lock, or recirculation through a filter within it. The advantage gained will depend upon the recirculation rate, the time during which recirculation is applied, if not continuous, and the type of airlock involved.

For a lock with positive supply v_L in addition to recirculation at a volumetric rate R, applied on entry to the lock, transfer on passage in the forward direction is reduced to approximately $mw(w/V_L + v_R/(R+v_L))$ for rapid passage through and to $mwv_R/(R+v_L)$ if the dwell time is sufficiently long. Return through the lock will also transfer $mwv_R/(R+v_L)$ in the same direction irrespective of dwell time, i.e. $v = mw(w/V_L + 2v_R/(R+v_L))$ for rapid passage, dropping to $2mwv_R/(R+v_L)$ with sufficient dwell time. Transfer in the reverse direction, u, will be determined by replacing v_R by v_C in the above formula. As an example, if recirculation at 2000 m.^{3}/hr. is applied to a lock of volume 10 m.^{3} with $v_C = v_R = 50$ then the transfer on rapid passage is $mw(0{\cdot}1w + 100/2100) = mw(0{\cdot}1w + 0{\cdot}048)$. With this rate of ventilation the effects of sedimentation are negligible and the residual concentration in the lock is reduced to about 12 % after a dwell of 0·01 hr. Transfer on passage is then reduced to $mw(0{\cdot}012w + 0{\cdot}048)$ which is insignificantly different from that resulting from indefinitely long dwell time, i.e. $0{\cdot}048mw$, since w is likely to be of the order of 1 m.^{3} (see below). The effects of recirculation applied continuously are identical with those obtained with operation only on entering the lock.

If there is extract from the lock, continuous recirculation will reduce transfer to $u = 2mwu_R(R+u_L)$ and $v = 2mwu_C/(R+u_L)$ irrespective of dwell time. If the recirculation is switched on only on entry into the lock, the recirculation will have no effect on transfer by rapid passage, and the transfer rate can be no more than halved even with prolonged dwell time.

By the use of directed downward flow ventilation in the airlock nearly 100 % clearance could be obtained in a single air pass. At a velocity of 0·5 m./sec. this would take little more than 5 sec. If, however, the floor area of the airlock were 5 m.^{2} the air supply rate would then need to be some 9000 m.^{3}/hr. or 150 m.^{3}/min.

Calculated transfer values

The relationships discussed above may be applied to an infinite variety of possible systems. For illustrative purposes the results obtained for a series of variations on a single layout, using plausible values for the various parameters, have been set out in Table 1. The assumptions of complete and uniform mixing within each space and of uniform regular entries and exits to the several rooms are manifestly approximations so that the precise numerical values must be treated with reserve.

In addition to selecting values for the physical dimensions of the system and for the rates of ventilation, it has also been necessary to assume values for m, the number of passages in each direction between a room and the communications area and for w, the volume of air exchanged between the two sides of a door when it is opened and closed in quick succession.

One study of a two-bed isolation room (Lidwell & Towers, 1970) has shown an

Table 1. *Calculated reductions in exposure to airborne particle transfer*

Condition	u	v	α	β
A. Balanced supply (no airlocks)	1000	1000	11 (6)	2 (1·7)
	500	500	28 (11)	4 (2)
	200	200	80 (34)	7·5 (4)
	100	100	230 (90)	19 (9)
	50	50	800 (270)	60 (21)
B. Directional flow (no airlocks)	500	10	$0{\cdot}9 \times 10^3$	65
	200	10	$1{\cdot}4 \times 10^3$	100
	60	10	$3{\cdot}3 \times 10^3$	220
	10	500	$0{\cdot}6 \times 10^3$	73
	10	200	$1{\cdot}0 \times 10^3$	100
	10	60	$2{\cdot}9 \times 10^3$	220
C. Airlocks				
Unventilated:				
(*a*) balanced	[1·5	1·5	$6{\cdot}7 \times 10^5$	$4{\cdot}7 \times 10^4$]
(*b*) with directional air flow	50	1·5	$2{\cdot}0 \times 10^4$	$1{\cdot}4 \times 10^3$
	1·5	50	$2{\cdot}0 \times 10^4$	$1{\cdot}4 \times 10^3$
(All with allowance for sedimentation in the airlock)				
Ventilated:				
input or extract	5	5	$6{\cdot}0 \times 10^4$	$4{\cdot}2 \times 10^3$
with allowance for sedimentation	2·9	2·9	$1{\cdot}8 \times 10^5$	$1{\cdot}3 \times 10^4$
D. Airlocks with recirculation				
Input ventilation				
(*a*) rapid passage	0·75	0·75	$2{\cdot}7 \times 10^6$	$1{\cdot}9 \times 10^5$
(*b*) 0·01 hr. dwell	0·24	0·24	$2{\cdot}6 \times 10^7$	$1{\cdot}8 \times 10^6$
Extract ventilation				
(*a*) rapid passage	2·9	2·9	$1{\cdot}8 \times 10^5$	$1{\cdot}3 \times 10^4$
(*b*) 0·01 hr. dwell	1·5	1·5	$6{\cdot}7 \times 10^5$	$4{\cdot}7 \times 10^4$
Directed ('laminar') flow	$\rightarrow 0$	$\rightarrow 0$	$\rightarrow \infty$	$\rightarrow \infty$

The table shows values of the ratio, α, of particle concentration in the source room to that in other patient rooms, and the ratio, β, of the concentration if all the rooms were combined into one space with uniform mixing throughout to the concentration in non-source patient room for the particular system, i.e. the larger the figure the better the isolation. u and v, the air flows from each room into the communications area and in the reverse direction are given in m.³/hr.

The following values have been used in the evaluation of α and β, particle settling velocity 0·3 m./min. Ten patient rooms each with floor area 15 m.² and air input at 300 m.³/hr. (this corresponds to a ventilation rate of 6/hr. for a room height of 3 m.). Settling in these rooms is then equivalent to an additional clean air supply of $0{\cdot}3 \times 60 \times 15 = 270$ m.³/hr., hence $v_1 = 300 + 270$ and has been taken as 600 m.³/hr. The communications area covers 80 m.² with an air input of 1200 m.³/hr. (this corresponds to a ventilation rate of 5/hr. for a ceiling height of 3 m.). Settling in this area is equivalent to an additional clean air supply of $0{\cdot}3 \times 60 \times 80 = 1440$ m.³/hr., hence $v_2 = 1200 + 1440$ and has been taken as 2500 m.³/hr. Entries and exits to and from each room have each been taken as 5/hr. ($m = 5$) and 1 m.³ of air has been assumed to be exchanged across the door opening each time a door is opened and shut. The volume of an airlock has been taken as 10 m.³ with a floor area of 4 m.² and with 100 m.³/hr. input or extract when appropriate. When the airlock is ventilated the air has been considered as delivered to or taken from both sides of the lock in equal amounts. Recirculation has been taken as 2000 m.³/hr. and assumed to come into operation only on entering the locks. The figures in parentheses in group A relate to conditions of negligible ventilation supply to the system. The values for the unventilated airlock have been enclosed in square brackets since this condition is practically unrealizable unless sealed doors are used.

average of 360 openings of the room door, entries or exits, per 24 hr. or 15/hr. ($m = 7{\cdot}5$). Observations on single rooms in a burns unit (Hambraeus & Sanderson, 1972) gave a figure of about half this for a burned patient and rather more for an amputee. It would therefore seem reasonable to take a value of 10 exits or entries/hr., i.e. $m = 5$, for the number of passages/hr. in each direction.

There do not appear to be any published data available on the exchange of air through a doorway on opening and closing the door. Since the volume swept out by a hinged door of conventional size is of the order of 1 m.^{3} this figure has been taken as an approximation for the purposes of calculation.

Unpublished experiments carried out recently by the Building Services Research Unit of Glasgow University (W. Whyte & B. Shaw, personal communication) indicate values of 0·5 m.^{3} for the air transfer on opening a swing door of conventional dimensions. The figure is substantially less for a sliding door, but the large gaps around such doors may lead to significant air exchange across the closed door unless there is sufficient directed air supplied to swamp reverse convectional air flow.

In general hospitals in Britain the doors of patient rooms without airlocks are commonly found to be left open for considerable periods of time (Baird, 1969; N. Foord & O. M. Lidwell, to be published). There have been several published studies of air exchange through open doors or similar apertures as a consequence of small temperature difference (Wolf, Harris & Hall, 1961; Brown, Wilson & Solvason, 1963; Kethley, 1966; Ma, 1965). Unfortunately the numerical values given by the different authors are not in agreement. They range from 40 to 1100 m.^{3}/hr. for a temperature difference of 1° C. across a 2 m. × 1 m. doorway, with as much as 350 m.^{3}/hr. for only 0·1° C. temperature difference in one instance. Since the air flow, theoretically, is proportional to the square root of the temperature difference quite high air flows may result from very small differences and values of between 100 and 500 m.^{3}/hr. seem not unlikely in practice.

DISCUSSION

With balanced or natural ventilation systems (group A, Table 1) values of α might be expected to lie between 10 and about 250. The actual advantage of subdivision compared with the exposure expected if all the patients were in a single room, given by the value of β, is, as explained earlier, much less, a reduction of between 2- and 20-fold in exposure to airborne particles. Comparison with the limited experimental results available, given in Table 2, shows a reasonable correspondence with the calculated figures. The degree of isolation obtained was, as would be expected, markedly less and the probable extent of air exchange between the parts of the ward much greater in the first situation referred to in Table 2. In this hospital the ward was partitioned but the patient areas opened directly off the communications corridor, being separated from it by no more than a low wall less than 1 m. high.

The introduction of a substantial directional flow to swamp the convectional exchange (group B, Table 1) raises the value of α to around 10^3 and β to about 100

Table 2. *The exposure to airborne particle transfer observed in some hospital situations*

Ref.	System	Conditions	Tracer	v_1	v_2	u or v	α
			A. Natural and balanced				
1	Partitioned ward	Natural ventilation	*Staph. aureus*	—	—	(1400)	5
2	Divided ward	Natural ventilation	*Staph. aureus*	—	—	(600)	10
3	Divided ward	Natural ventilation	*Staph. aureus*	—	—	(200)	40
4	Divided ward	Natural ventilation	*Staph. aureus*	—	—	(400)	18
5	Divided ward	Part mechanical ventilation	Nitrous oxide	50–200	4000	100–170	70–80
5	Divided ward	Air conditioned	Nitrous oxide	700	4000	300	80
			B. Directional flows				
6	Isolation unit	Positive rooms	*Staph. aureus*	350	?1500	20	500
7	Divided ward	Positive rooms	*Staph. aureus*	800	2500	*	14
7	Divided ward	Positive rooms	Freon gas	800	2500	50	100
7	Divided ward	Positive rooms	Pot. iodide particle	800	2500	50	400
			C. Airlocks				
8	Isolation unit	Extract airlocks	Pot. iodide particle	200	1100	2·9	$3{\cdot}4 \times 10^5$
8	Isolation unit	Extract airlocks	Pot. iodide particle	200	1100	17	$9{\cdot}6 \times 10^3$

The table shows the observed values of α, the ratio of the particles concentration in the source room to that in other patient rooms. The values of v_1, the ventilation supply to a patient room and v_2, that to the communication area, both given in m.^{3}/hr., are usually only rough estimates based on limited information. Values of u or v, also in m.^{3}/hr., have been deduced from the above using equation 5. For the balanced and natural ventilation system u and v are assumed equal. For references 6 and 7 u has been taken as 200 and 400 m.^{3}/hr. respectively and the values given in the 7th column are those calculated for v. Where the tracer is particulate v_1 and v_2 have been doubled to allow for sedimentation (this is true if $s = 0{\cdot}3$ m./min., $h = 3$ m. and ventilation is at 6 changes/hr.) before applying the formula. No estimates of v_1 or v_2 can usefully be made for the first four situations listed. The values of u and v given in parentheses are deduced from those given in Table 1 for the no ventilation condition.

The two values of u, v and α for reference 8 refer to 10 and 60 entries/exits per hr. respectively ($m = 5$ and $m = 30$).

* The discrepancy between the value of α in this row and that for the same system with a similar-sized tracer particle in the row below, suggests that the micro-organisms reached the non-source rooms by other routes than air transfer.

References. 1, Lidwell *et al.* (1971); 2, Lidwell *et al.* (1966); 3, Williams (1967); 4, Edmunds (1970); 5, Baird (1969); 6, Williams & Harding (1969); 7, Foord & Lidwell (to be published); 8, Hambraeus & Sanderson (1972).

(this assumes the figure of 10 entries or exits to or from the room in each hour transferring 1 m.^{3} of air with each door opening). The observed ratios (group B, Table 2) are not quite as high as this and correspond to transfer against the direction of air flow of 20–50 m.^{3}/hr., 2–5 times the assumed figure. This would suggest that the directional flows were inadequate to prevent reverse flow. The insertion of an airlock between the room and the corridor, group C, further increases the ratio α to between 10^4 and 10^5, on the same assumptions of the frequency of door opening and the extent of air transfer associated with this. The calculated

performance for a ventilated airlock is independent of any delay or dwell on passing through the lock. Extract from the lock has the advantage of reducing dispersal of any airborne contamination generated within it. The one set of figures available in Table 2 show quite close agreement with the theoretical calculations and are presented in greater detail in the next paper (Hambraeus & Sanderson, 1972).

By using high volume recirculation in an airlock it should be possible to increase the value of α to very high values indeed but these could be realized in practice only if transfer of particles by other means than free air, for example on clothes or in the respiratory system, was kept to similarly low levels.

The clinical significance of these calculations and observations is quite uncertain since in no situation have we precise knowledge of the risk of infection by the airborne route as a function of exposure. It is, however, desirable that whenever studies are made of the clinical value of an isolation system measurements of the airborne particle transfer within it should be made under working conditions and that, if a high level of air isolation is considered desirable, the design should be such as to produce this. In order that any system may function effectively the actual air volumes and balances must be stable and correspond to those called for by the design. This is not generally the case, and most systems that I have examined have been both variable and divergent from their design intentions, for example the isolation unit referred to in reference 8 (Table 2) was designed for an input of 200 m.^{3}/hr. to each of the patient rooms with an extract of 100 m.^{3}/hr. from the airlock, supplied as to 50 m.^{3}/hr. from the room and 50 m.^{3}/hr. from the corridor. The value of α given in the table was obtained as between rooms where these flows were, at least approximately, maintained. However, out of 27 observations of the air flow around the doors into the air locks made on six separate days the *direction* of flow was reversed around one or other door on no fewer than 11 occasions. Hambraeus & Sanderson (1972) report similar observations. It must always be difficult to maintain the balance between flows as small as the above and if it is to be achieved some stabilizing factor, for example a relatively high-pressure drop across a filter or control orifice, must be included in the system. In addition, visual or other indicators of the flow directions are called for to ensure maintenance of the design conditions.

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The control by ventilation of airborne bacterial transfer between hospital patients, and its assessment by means of a particle tracer

III. Studies with an airborne-particle tracer in an isolation ward for burned patients

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SUMMARY

Airborne-particle transfer has been studied in a burns unit using potassium iodide particles. The observed rates of transfer were in good agreement with the values predicted by a theoretical model.

An estimate of the average transfer between rooms under conditions of normal activity and with correctly functioning ventilation showed that the isolation system was highly efficient, the proportion transferred being probably less than 1 in 10^5. However, the ventilation often did not function as designed and under these conditions the efficiency was reduced by a maximum of a factor of ten. These rates of transfer do not seem great enough to account for the high rate of cross-infection found in this unit.

INTRODUCTION

Much effort has been expended in recent years in devising ventilation systems to reduce the airborne spread of bacteria in hospital wards, but methods for evaluating the efficiency of these are not altogether satisfactory and have only occasionally been attempted. In a preceding paper (Foord & Lidwell, 1972) a method has been described for studying airborne particle transfer between parts of a ward by use of potassium iodide particles. We now present the results of a series of experiments using this method in an isolation ward for burned patients at Akademiska sjukhuset, Uppsala. In spite of a complete ventilation system, including airlocks, earlier studies have revealed a considerable amount of cross-infection between patients in the ward (A. Hambraeus, to be published). The purpose of the investigation was to determine the effectiveness of the ventilation system and hence the role of airborne bacteria as a possible cause of cross-infection. Comparison could also be made with the theoretical analysis of isolation system elaborated in the second paper of this series (Lidwell, 1972).

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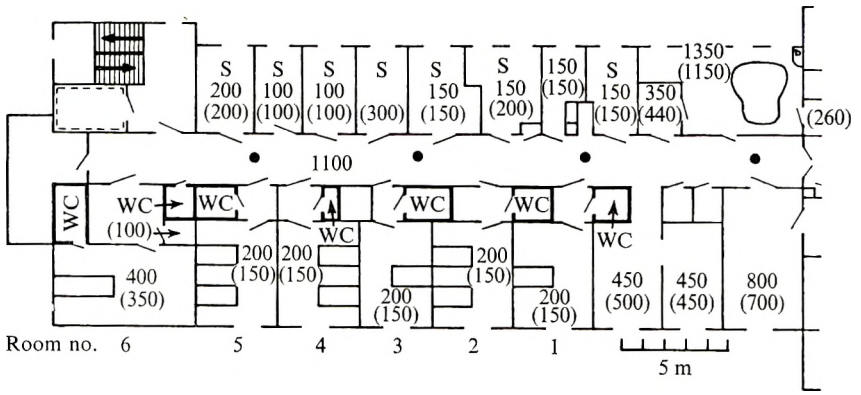


Fig. 1. Plan of the ward. Input ventilation is shown for each space as m.³/hr., extract volumes are given in parentheses, an additional 100 m.³/hr. was extracted from each of the patient room W.C.'s, except no. 6. The sampling sites, when room 1 was the source room, are indicated by ●; S = service room.

MATERIALS AND METHODS

Ward design and ventilation

Fig. 1 shows the plan of the isolation ward. It is entered via an airlock with double doors and a passage runs down the middle of the ward. There are five patients' rooms of similar dimensions and a sixth larger room containing an air bed. All of these have individual airlocks and they are situated along one side of the passage. On the opposite side of the passage are service rooms and the bathroom. The bathroom has three doorways; one of these opens directly to the passage for bed transport, and the other two open via airlocks; one into the passage (I) and the other into the ward airlock (II). The designed ventilation input and extract are shown in each part of the ward (the extract is in brackets). The net result is a near-balanced ventilation with little or no other air flow between the ward and the exterior.

Fig. 2 shows the air flow as designed for a typical patient room. The air input to the room is 200 m.³/hr. (which approximately equals 4 air changes/hr.). Of this input, 150 m.³/hr. is extracted directly from the room, the remaining 50 m.³/hr. passes into the airlock. A further 50 m.³/hr. is drawn from the passage into the airlock making up the total extract from the W.C. of 100 m.³/hr.

The ventilation of the bathroom is shown in Fig. 3. The designed input is 1350 m.³/hr. and the extract is 1150 m.³/hr. Airlock I has an input of 350 m.³/hr. and an extract of 440 m.³/hr., this results in a net deficit in airlock I of 90 m.³/hr. which is supplied as 45 m.³/hr. from both the bathroom and the passage. The extract from airlock II is 260 m.³/hr., this is provided as 130 m.³/hr. from both the bathroom and the ward airlock. This leaves a net surplus of air supply in the bathroom of (200–175) m.³/hr. = 25 m.³/hr. which escapes into the passage beneath the direct door.

Titanium tetrachloride smoke was used to determine the direction of air flow beneath the doors.

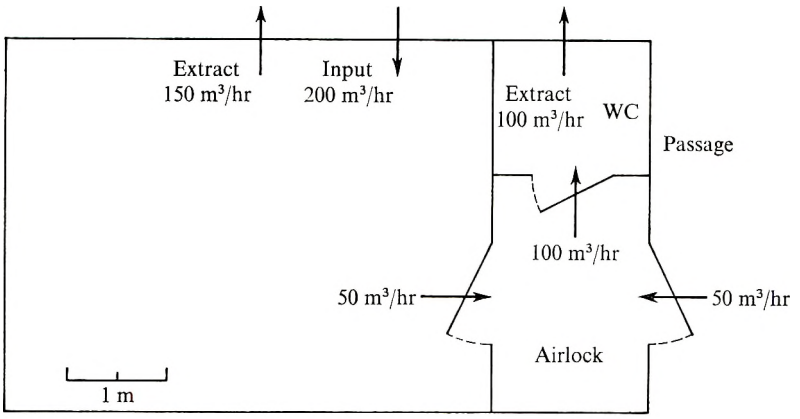


Fig. 2. Diagram showing the design ventilation of patient rooms 1–5.

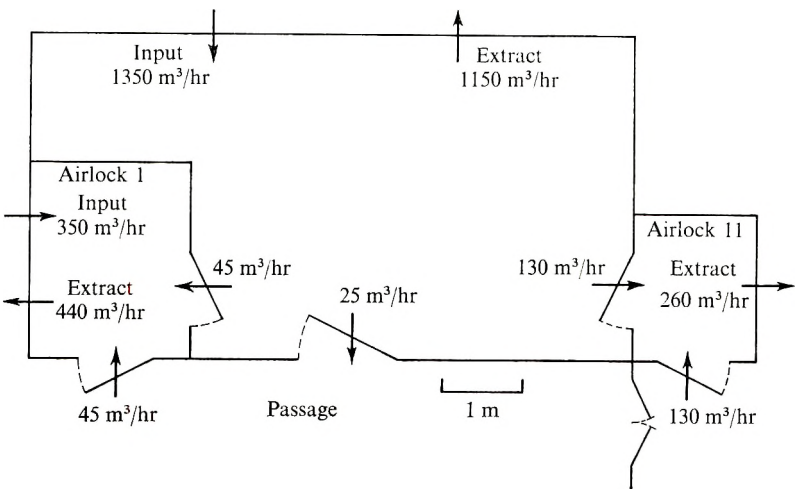
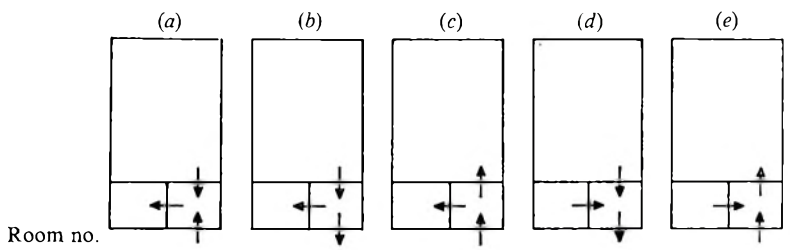


Fig. 3. Diagram showing the design ventilation of the bathroom.



Room no.	(a)	(b)	(c)	(d)	(e)
1	30	2	—	—	—
2	24	8	—	—	—
3	8	23	1	—	—
4	15	9	2	4	2
5	15	17	—	—	—
6	19	2	11	—	—
No. of observations	111	61	14	4	2

Fig. 4. Ventilation patterns observed for the patient rooms. (*a*), directions of air flow as designed; (*b*), (*c*), (*d*) and (*e*), other observed air-flow patterns. The table shows the number of times the respective pattern was observed in the specified room.

Over a preliminary period of 32 days it was found that the designed ventilation was not maintained at all times and the frequency of variation is shown in Fig. 4. It can be seen that the commonest fault was a reversal of air flow beneath the door between the airlock and the passage, thus allowing escape of air from the room to the passage. We therefore included this type of ventilation in our experiments and all references to incorrect ventilation refer to this. The direction of air flow was checked before every experiment.

Particle generation

A spinning disk particle generator was used throughout the experiments. The rotation rate of the disk was 60,000 rev./min. and a main air-flow rate of 60 ft.^{3}/min. (1·7 m.^{3}/min.). The satellite air flow removes smaller satellite particles from the main air flow and this was found to operate satisfactorily at 6 ft.^{3}/min. (0·17 m.^{3}/min.).

Potassium iodide dissolved in methyl alcohol and supplied to the spinning disk at a rate of 1 ml./min. gave rise to a particle production rate of c 10^7 particles/min.

Sampling methods

Air sampling was carried out by trapping the particles on 2·5 cm. diameter membrane filters (Millipore Filter Corp.) of 3 μm. pore size. Two types of sampler were used.

(*a*) Where the KI particle concentrations were high, known volumes of air were sucked through the millipore filters. Open-ended plastic filter holders were used (Foord & Lidwell, 1972). Owing to the high air-flow resistance of the filters the maximum air flow obtainable with the pumps which were available was 10 l./min. This was suitable for use with KI particle concentrations of more than 5/l.

(*b*) In situations where there were low particle concentrations the centripetal samplers described by Foord & Lidwell (1972) were used. These were designed to have a collection efficiency of 100 % at flow rates above 60–70 l./min. for 13 μm. equivalent diameter particles (i.e. particles which have the same sedimentation rate as a 13 μm. particle of specific gravity 1). A suction pressure of 20 cm. water gave a flow rate of 100 l./min. and under these conditions only 3 % of the air flow passed through the filter.

Staining. After particle collection the filters were immediately stained with 1 % palladium chloride in 0·1 % HCl.

Counting. Particle counting was performed with the aid of a low-power plate microscope and a grid. Up to 200 particles were counted.

Determination of particle size

Two ways of determining particle size are available:

(*a*) By direct microscopy of particles deposited on a silicone grease coated slide.

(*b*) By calculation of the sedimentation rate and hence the equivalent particle diameter by Stokes Law ($s \triangleq 0{\cdot}002d^2$), where the sedimentation rate, s, is in units of m./min., and the diameter, d, is in units of μm.

Methods of calculating the sedimentation rate of airborne particles are described

in 'Studies in Air Hygiene' (Bourdillon, Lidwell & Lovelock, 1948). Two independent methods of determining the sedimentation rate were used. First, from the die-away rate (K), which is the logarithmic rate at which particles disappear, $K \triangleq$ slope $\times$ 138/hr. In an unventilated room K is due solely to sedimentation and thus $K = sA/V$, from which the equivalent diameter can be found (A is the area onto which sedimentation is occurring and V is the volume of air from which sedimentation occurs).

Alternatively, the sedimentation rate can be calculated from the number of particles sedimenting onto a known area when the number of particles in a unit volume of air is known. This can be represented as: no. of particles sedimenting $= s \times$ the concentration of particles in the air.

EXPERIMENTAL DESIGN

Preliminary studies to find an appropriate particle size

A small sealed room was used in which particles were generated. Different size particles are produced by varying the concentration of the potassium iodide solution fed to the spinning disk and the concentrations used were 2·0, 3·5 and 5·0 %. Particles were generated for 20 min. and 1 l. air samples were taken at 5 min. intervals during this time and for the 20 min. after particle generation had ceased. In addition filters were exposed on the bench for the last 5 min. of particle generation. The concentrations of KI gave particles with the following sedimentation rates: 2·0 % = 0·15 m./min.; 3·5 % = 0·23 m./min.; 5·0 % = 0·31 m./min.

A 5 % potassium iodide solution was used for the transfer experiments and this gave particles with a sedimentation rate close to the median found for airborne particles carrying *Staphylococcus aureus* in hospital wards (Noble, Lidwell & Kingston, 1963).

Studies on the transfer of particles from the room to the passage

Since the earlier study had shown the variability of air flow in the ward, it was decided to perform experiments using both the correctly ventilated air locks and also the commonest type of ventilation defect (i.e. that in which the air flow beneath the door between the passage and the airlock was reversed).

The generator was situated in the room chosen and adjusted to give a concentration of 500–1000 particles/l. in the room. There was no activity for the first 30 min. and then, for the following 30 min., one walk from the room to the passage was performed every second min. The dwell time in the airlock was 30 sec. Throughout the experiment samples were taken at the following sites; (*a*) in the room, samples taken at 5 min. intervals; (*b*) in the airlocks, samples were taken at 5 min. intervals during the period of no activity; (*c*) across the passage 2 m. from the room door, 7·5 m. up the passage, 7·5 m. down the passage, and at 15 m. either up or down the passage according to the room in use. These sites are shown in Fig. 1 for the experiments when the generator was in room 1. One 10 min. sample was taken between 20 and 30 min. and then during activity samples were taken at 5 min. intervals.

Particle transfer from passage to room

The generator was situated in the passage about 3 m. from the door of the room to be investigated. The periods of activity and non-activity were the same as before, as was the type of activity. Samples were taken at 1 m. from the door (at 5 min. intervals throughout the experiment), in the airlock (at 5 min. intervals during the period of no activity), and in the centre of the room where one 10 min. sample was taken between 20 and 30 min. and then 5 min. samples were taken continuously during the period of activity. Correct and incorrectly ventilated airlocks were used.

Transfer of particles from the bathroom to the passage

The generator was situated in the bathroom and there was no activity for the first 30 min. of generation, for the next 30 min. there was activity through the airlock I at the rate of one walk to the passage and back every 2 min. There was then a period of non-activity for 15 min. and for the last 20 min. there was activity through the direct door to the passage at the same rate as before. During this time samples were taken in the same six sites as they were in the room to passage experiments and the sampling times were also the same. The only exceptions to this were that no samples were taken in the airlock during the second period of non-activity and only for the last 10 min. of this period in the passage. During the last period of activity samples were taken once every 5 min. in the passage and in the bathroom.

Transfer of particles from passage to the bathroom

Particles were generated at about 3 m. from the bathroom doors which opened into the passage. Samples were taken between these two doors (about 1 m. from each). Samples were also taken in the airlock I and in the bathroom. The activity was the same as that in the bathroom to passage experiments and the sampling was the same as that in the passage to room experiments, except that no samples were taken in the airlock during the second period of non-activity. In the last two experiments correctly and incorrectly ventilated airlocks were used.

In addition to the above samples, blank samples were taken at each site before the start of each experiment.

Calculation of results

In all the experiments the particle concentration at any site started at zero and rose to a steady value in a short time. The steady values were determined by inspection of the data. For the source site, equilibrium was usually achieved within 10 min. of the start of generation, and at the receiving site a steady state was usually achieved after 10–15 min. of walking (depending on the site of sampling). The average particle concentration during the steady-state period was used in all calculations.

The behaviour of ventilation systems of the type under investigation has been studied theoretically by Lidwell (1972), making a number of simplifying assump-

tions. The analysis which follows is based on this study and has been carried out in collaboration with him.

The ratio α of the number of particles at the source to that at the receiving site gives a measure of the effectiveness of the system in protecting the patient from airborne infection, i.e.

$$\alpha = \frac{\text{Concentration of particles in the source room}}{\text{Concentration of particles in the receiving room}}$$

(large values of α indicate a high degree of protection).

Since the passage intervenes between the patient rooms, transfer of particles may be conveniently analysed by breaking the process down into two stages: (1) transfer from the source room to the passage; (2) transfer from the passage to the receiving room.

α' and α'' are used as symbols for these ratios, i.e.

$$(1) \quad \alpha' = \frac{\text{Concentration of particles in the source room}}{\text{Concentration of particles in the passage}},$$

$$(2) \quad \alpha'' = \frac{\text{Concentration of particles in the passage}}{\text{Concentration of particles in the receiving room}}.$$

Similar ratios can be calculated for bathroom to passage transfer and vice versa. Thus

$$\alpha' \text{ (bathroom)} = \frac{\text{Concentration of particles in the bathroom}}{\text{Concentration of particles in the passage}},$$

and

$$\alpha'' \text{ (bathroom)} = \frac{\text{Concentration of particles in the passage}}{\text{Concentration of particles in the bathroom}}.$$

The theoretical calculations predict that

$$\alpha \doteq v_1 v_2/uv, \quad \alpha' \doteq v_2/u, \quad \alpha'' \doteq v_1/v,$$

where v_1 is the total rate of ventilation (both real and apparent due to sedimentation) in each patient room and v_2 is the total rate of ventilation in the passage (including the effects of sedimentation). v is the rate of air movement from the passage to the room, and u is the rate of air movement from the room to the passage. In addition to any continuous air flows due to incorrect ventilation conditions u and v will include air transferred by movement through the doors. This is related to the frequency of movement through the doors (m) and the amount of air swept through the door in the direction of movement on each occasion (w).

The ventilation input to the patient rooms is 200 m.^{3}/hr. The apparent ventilation due to particles sedimenting at 0·3 m./min. onto the floor area of 16 m.^{2} = $16 \times 0{\cdot}3 \times 60 = 300$ m.^{3}/hr.

Similarly in the passage, the ventilation input is 1100 m.^{3}/hr. and the apparent ventilation when the floor area is 90 m.^{2} is 1600 m.^{3}/hr. The input to the bathroom is 1350 m.^{3}/hr. The apparent ventilation due to a floor area of 30 m.^{2} is 550 m.^{3}/hr.

Hence $v_1 = 200 + 300 = 500$ m.^{3}/hr.

and $v_2 = 1100 + 1600 = 2700$ m.^{3}/hr.

and v_1 (bathroom) $= 1350 + 550 = 1900$ m.^{3}/hr.

For a purely extract-ventilated airlock with air drawn equally from both sides it can be shown that $u = v = mw$ (Lidwell, 1972). However, the actual transfer of particles will be less than this because of the loss of particles by sedimentation within the airlock. The room airlock has a floor area of 4 m.^{2} and an extract ventilation of 100 m.^{3}/hr. The proportion of particles with a settling velocity of 0·3 m./min. lost by sedimentation is then 0·42. If the air was drawn equally from both sides then the effective value of u and v for the transport of particles becomes $(1–0{\cdot}42)mw = 0{\cdot}58mw$.

The bathroom airlock had an air supply of 350 m.^{3}/hr. The extract of air from the bathroom airlock was 440 m.^{3}/hr. Assuming that the difference was drawn equally from both sides 45 m.^{3}/hr. will have come from the passage and 45 m^3./hr. from the bathroom itself. As a consequence $u = v = 45/440 \times 2mw = 0{\cdot}2mw$. The floor area was 5 m.^{2} so the proportion lost by sedimentation was, making the same assumptions as earlier, $(60 \times 0{\cdot}3 \times 5)/(60 \times 0{\cdot}3 \times 5 + 440) = 0{\cdot}17$. The effective values of u and v for the transport of particles through the bathroom airlock are then given by $u = v = (1 - 0{\cdot}17) \times 0{\cdot}2mw = 0{\cdot}19mw$.

The experimental value of m was 30/hr. and for the purposes of calculation we have assumed that $w = 1$ m.^{3}.

For the room airlock therefore the predicted value of u and v during the experiments was $30 \times 0{\cdot}58 = 17$ m.^{3}/hr. and for the bathroom airlock $30 \times 0{\cdot}17 = 5$ m.^{3}/hr. For the direct bathroom door the corresponding value would be 60 m.^{3}/hr. (there is no sedimentation loss to allow for in this instance).

Substituting these values in the equations for α' and α'' gives the following results:

(1) room to passage $= \alpha' = \dfrac{v_2}{u} = \dfrac{2700}{17} = 160$;

(2) passage to room $= \alpha'' = \dfrac{v_1}{v} = \dfrac{500}{17} = 29$;

(3) room to room $= \alpha = \alpha' \times \alpha'' = \dfrac{2700 \times 500}{17 \times 17} = 4{\cdot}7 \times 10^3$;

(4) bathroom to passage $= \alpha'$ (bathroom) $= \dfrac{2700}{5} = 540$;

(5) passage to bathroom $= \alpha''$ (bathroom) $= \dfrac{1900}{5} = 380$;

(6) bathroom to room $= \alpha = \dfrac{2700}{5} \times \dfrac{500}{17} = 1{\cdot}6 \times 10^4$;

(7) room to bathroom $= \alpha = \dfrac{1900}{5} \times \dfrac{2700}{17} = 6{\cdot}1 \times 10^4$.

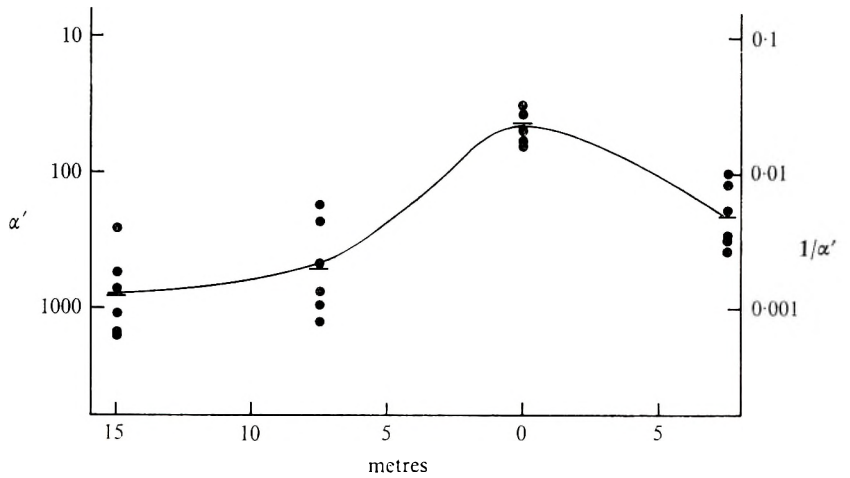


Fig. 5. Distribution of particles along the passage. Source in room 1 with correct ventilation and activity at 30 entries and exits/hr. Right-hand scale is the ratio of the particle concentration in the passage to that in the source room, i.e. $1/\alpha'$. The points show the approximately steady values attained in the individual experiments. The short horizontal bars are the log mean values as given in Table 1.

These values are calculated for the experimental movement through the doors of 30/hr. in each direction. The values of α' and α'' for correctly ventilated airlocks are inversely proportional to m and α is inversely proportional to m^2.
For other values of m

$$\alpha'_m = \alpha'_{30}\,(30/m), \quad \alpha''_m = \alpha''_{30}\,(30/m), \quad \alpha_m = \alpha_{30}\,(30/m)^2.$$

If, however, the ventilation is unbalanced so that there is air flow between passage and room independent of movement of persons through the doors the transport of particles by this air flow is unaffected by a change in the value of movement through the door and

$$\frac{1}{\alpha'_m} = \frac{1}{\alpha'_0} + \left(\frac{1}{\alpha'_{30}} - \frac{1}{\alpha'_0}\right)\frac{m}{30},$$

and similarly for α''.

Where α'_m denotes α' when the movement through the door is m/hr., α'_0 is the value of α' when there is no activity and α'_{30} corresponds to the experimental value of α', when there are 30 entries and exits/hr. through the door, the number employed in the experiments.

RESULTS

Fig. 5 shows the way in which the ratio of particles in the room and passage vary with increasing distance of the sampling site from the room; it also shows variability of the estimates obtained in the several experiments. The values used were obtained from room to passage transfer experiments with a correct ventilation.

The mean values of α' and α'' for the various sampling positions for all the experiments performed are presented in Table 1. The values are log means of at least five experiments. The table also shows the log mean values of α' over all the positions. The corresponding values of α are calculated from these values and also shown in Table 1. As can be seen from the table there is no detect-

Table 1. *Particle transfer within the Burns Unit. Avd. 79A*

Nos.	Transfer	Ventilation	Activity	α' at sampling position: 7·5a	2	7·5b	15b	α' Mean	α''	$\alpha = (\alpha' \times \alpha'')$†: To P-room	To B-room
1–4	Patient room/ passage	Correct	None	$> 10^5$	$> 10^4$	$> 10^5$	$> 10^5$	$(> 10^5)$	$(> 10^5)$	$> 10^9$	—
		Correct	In/out through airlock	204	45	505	818	245	39	$9{\cdot}6 \times 10^3$	—
		Faulty	None	248	58	226	1200	251	$(> 10^5)$	$> 2{\cdot}5 \times 10^7$	—
		Faulty	In/out through airlock	71	20	46	780	83	66	$5{\cdot}5 \times 10^3$	—
5–10	Bathroom/passage	Correct	None	88	22	297	1050	155	$(> 10^5)$	$> 1{\cdot}5 \times 10^7$	$> 10^{10}$
		Correct	In/out through airlock	44	19	173	562	96	215	$3{\cdot}8 \times 10^3$	$5{\cdot}3 \times 10^4$
		Correct	In/out through direct door	39	10	292	741	96	63	$3{\cdot}8 \times 10^3$	$1{\cdot}5 \times 10^4$
		Faulty	None	38	21	151	751	98	$(> 10^5)$	$> 1 \times 10^7$	$> 2{\cdot}5 \times 10^7$
		Faulty	In/out through airlock	39	21	48	480	66	400	$4{\cdot}3 \times 10^3$	$3{\cdot}3 \times 10^4$
		Faulty	In/out through direct door	31	15	79	202	52	66	$3{\cdot}4 \times 10^3$	$5{\cdot}5 \times 10^3$

The values of α' and α'' are, in each case, the log mean values of 5–7 experiments. α' mean = α' averaged over all sampling positions, log mean of 4 values.

† Left-hand column α values for transfer to a patient room; first 4 lines, from another patient room; last 6 lines from the bathroom. Right-hand column α values for transfer from a patient room to the bathroom. In all cases the same activity and type of ventilation is assumed to hold for both source and receiving room.

able transfer of particles from room to passage or passage to room when the ventilation functions correctly, i.e. α' is $> 10^5$, α'' is $> 10^5$. When a steady state has been achieved under activity α' is 245 and α'' is 39. For an incorrectly ventilated room with no activity α' is 251, i.e. about the same value as α' during activity for a correctly ventilated room, and during activity this falls to 83. However, α'' for an incorrectly ventilated room is 66, which is considerably higher than that for a correctly ventilated room. When the source room and receiving room are both correctly ventilated α is $9{\cdot}6 \times 10^3$; the corresponding α for incorrectly ventilated rooms is $5{\cdot}5 \times 10^3$. An incorrectly ventilated source room and a correctly ventilated receiving room gives an α of $83 \times 39 = 3{\cdot}2 \times 10^3$, and a correctly ventilated source room and an incorrectly ventilated receiving room gives an α of $245 \times 66 = 1{\cdot}6 \times 10^4$. The bathroom α' when the airlock is ventilating correctly is rather low, 155; this is due to the fact that particles are always passing from the bathroom into the passage underneath the direct door; because of this α' bathroom due to activity does not vary greatly between correct or incorrect ventilation or activity through the airlock or the direct door. α'' bathroom, however, is lower if the ventilation is correct than if it is incorrect, 215 as opposed to 400. There is also a difference between α'' bathroom if the activity is performed through the airlock or through the direct door. The value of α for transfer from the bathroom to a patient room during activity (entering and leaving the bathroom via airlock I) is $3{\cdot}8 \times 10^3$, when ventilation to rooms is correct. For transfer from a patient room to the bathroom under similar conditions the value of α is $5{\cdot}3 \times 10^4$.

DISCUSSION

As stated above there were two objectives in this work, to obtain an estimate of the degree of protection from airborne cross-infection provided by the ventilation system used in this ward and to compare the experimentally determined values for particle transfer with the values predicted by a theoretical analysis.

Owing to incomplete mixing of air in the passage there was a rapid fall in particle concentration with increasing distance from the door of the source room (Fig. 5). Because of this it is necessary to derive average values of α'. To estimate the average cross-infection risks of all the rooms, the logarithmic mean of α' at the different points was used since the risk of nasal acquisition (and hence the risk of infection) is probably more nearly related to the logarithm of the concentration of bacteria in the air (Lidwell, 1963; Lidwell *et al.* 1971) than to the arithmetic value. To compare the results with theory it is best to take an arithmetic average of the particle concentrations at the sampling points, i.e. the average of $1/\alpha'$ because the points are equidistant along the passage and the theoretical value of α' is based upon the assumption of complete mixing of the air. These values are given in Table 2. This average value was also used to calculate values of u ($u = v_2/\alpha'$). In deriving the experimental value of α' for the bathroom the particle transfer due to air passing beneath the direct door (i.e. $1/\alpha'$ with no activity) was subtracted from the particle transfer when there was activity. It is not possible to estimate the transfer when the ventilation is incorrect as the amount of extra transfer between the room and the passage (or vice versa) is not

Table 2. *Comparison of observed and predicted values (for designed ventilation)*

		α'	u	α''	v
Room (airlock)	Observed value	143	19	39	13
	Predicted value	160	17	30	17
Bathroom (airlock)	Observed value	170	16	215	9
	Predicted value	640	5	450	5
Bathroom (direct door)	Observed value	59	41	63	21
	Predicted value	45	60	23	60

The α' values have been derived as the arithmetic average of the particle concentrations at the different sampling positions, i.e. by summation and averaging of $1/\alpha'$, see text.

Predicted values of u and v assume a value of 1 m.^{3} for w, the volume of air transferred through a door on opening it, passing through and shutting it again.

known. For the room experiments, correlation of predicted and experimental values of α' and α'' is good.

For the bathroom the correlation is not so good but the ventilation of this room was certainly not in accordance with the specification and the leakage under the door into the passage meant that the values of α' had to be derived as a difference between two experimentally determined values. The general closeness of the estimates to the experimental results suggests that the theoretical model is broadly correct, but that errors are present in the estimation. Thus, the value of w is only an approximation and may be subject to considerable variation depending upon thermal difference across the door, size of the door and room, angle to which door is opened etc. Values of v_1 and v_2 were taken from the engineer's plan and in view of the variable ventilation state of the ward may have borne little relationship to the true values of v_1 and v_2. We were unfortunately unable to check the accuracy of the figures.

Experimental errors were due to two main contributions: (1) relatively small air samples were taken; (2) random air movements caused great variation in the particle concentration at different sites. A number of factors caused these air movements, amongst them were thermal currents, disturbances due to opening of doors, movements of staff and uncontrolled day to day variations in ventilation.

Estimation of the cross-infection risk

The estimated value of α for transfer of particles from one room to another with correct ventilation is $> 10^9$ when there is no activity (i.e. transfer of particles was undetectable) and $9{\cdot}6 \times 10^3$ when the rate of activity is 30 walks/hr. However, normal activity in the ward is much less than this and observations in this unit indicate a value of 3–5 door openings/hr., about half that found in a two-bed isolation room (Lidwell & Towers, 1969) and we have deduced that a reasonable estimate of the normal value is 5 walks/hr., i.e. $m = 5$. This leads to an α of $3{\cdot}4 \times 10^5$ between correctly ventilated rooms with normal activity. It would seem that this should provide good protection against cross-infection due to airborne contamination since a value of 1 colony forming unit (c.f.u.)/l. in a source room would give rise to a concentration of only 3×10^{-6} c.f.u./l. in the receiving room. This estimate of the concentration of bacteria-carrying particles in the room is

equivalent to only 0·15 c.f.u. in the whole volume of the room (5×10^4 l) or 1 c.f.u. inhaled every three weeks at an inspiration rate of 10 l./min. If 1/3 m.^{2} of body surface were exposed to contamination by settling and the airborne particles were of the size commonly found (settling velocity 0·3 m./min.) then the dose received in this way would be no more than 1 c.f.u. in 50 hr.

If the airlocks do not function correctly then the value of α will be different. The results of experiments upon the type of incorrectly ventilated airlocks investigated showed that in the absence of all activity α' for a room with an incorrectly ventilated airlock was as much as α' for a room with a correctly ventilated airlock when the level of activity was 30 walks/hr. However, α'' for such an incorrectly ventilated airlock during activity was higher than the corresponding value for a correctly functioning airlock. Thus the worst situation for which we can derive an estimate will be when the source room has an incorrectly ventilated airlock, and the receiving room has a correctly ventilated airlock. With a normal activity of 5 walks/hr the value of α would then be $4{\cdot}4 \times 10^4$. Thus if one airlock is functioning incorrectly the risk in all the other rooms is about ten times greater than under correct conditions, e.g. for a concentration in the source room of 1 c.f.u./l. a patient in a receiving room would inhale 1 c.f.u. every 2 days and about 5 c.f.u. might settle on 1/3 m.^{2} of exposed body surface each 24 hr.

There are two other possible ways in which contaminated air might reach the patient. Large amounts of bacteria may be dispersed during bathing in the bathroom and be transferred throughout the ward. Alternatively the patient might become infected in the bathroom with bacteria transferred from another patient room. In the first case there is continuous contamination of the corridor from the bathroom due to air passing beneath the direct door even when there is no activity through the correctly ventilated airlock. But it is greater when there is activity and when the airlock is ventilated incorrectly. The airlock, however, was usually ventilated correctly, and under normal conditions there was little activity through the airlock and none through the direct door. For the purposes of calculation we have assumed the activity through the bathroom airlock to be 5 walks/hr. although this is probably too high a value. This leads to a value of α for transfer from the bathroom to a patient room of $3{\cdot}9 \times 10^4$. In the second case the value of α for transfer from a patient room to the bathroom, again assuming 5 walks/hr. through the bathroom airlock, would be $1{\cdot}9 \times 10^6$ if the patient room ventilation functions correctly and $2{\cdot}4 \times 10^5$ if the airlock is incorrectly ventilated.

In neither of the above 3 cases is the risk of cross-infection much higher than the risk run by patients when in patient rooms, if one room with an incorrectly ventilated airlock is occupied by a disperser.

Conclusion

It would seem from the above results that, under normal conditions, the isolation system was highly effective at preventing the airborne transfer of particles and even when the ventilation was not operating correctly the particle transfer was very small. Since there has been a considerable cross-infection rate over the past years it would seem likely that other routes of infection have been responsible for this.

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Redox potential measurements for determining the disinfecting power of chlorinated water

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SUMMARY

The kill of *Escherichia coli* within 3 min. was studied in chlorine-demand-free water using sodium hypochlorite, monochloramine, dichloramine, halazone, chloramine T, cyanuric acid + sodium hypochlorite and cyanuric acid + monochloramine. The redox potential and the available chlorine were measured. The redox potential was found to be better correlated with the disinfecting property of the water than was the amount of available chlorine. For individual pure chlorine compounds, the measuring of available chlorine showed in general a somewhat better correlation with reduction of the bacteria than the redox potential showed.

INTRODUCTION

The most important disinfection agent for tap water, swimming-pool water and waste water is chlorine, which usually is added in the form of gaseous chlorine or hypochlorite. The activity of the chlorine compounds is expressed as the amount of pure gaseous chlorine with a corresponding oxidizing power (chlorine equivalents).

Most waters contain substances which can reduce chlorine (the 'chlorine demand' of the water). When ammonium ions are present inorganic chloramines are formed. The relative amounts of monochloramine, dichloramine and nitrogen trichloride depend upon the pH of the water. In addition, chlorine can form organic chloramines with nitrogenous organic compounds. These inorganic and organic chloramines are called combined residual chlorine. Chlorine which does not show a reaction to any of the above processes exists as hypochlorous acid and hypochlorite ion (free residual chlorine). Free and combined residual chlorine together make up the total residual chlorine in the water. Which of these chlorine compounds will be formed when chlorination is performed depends entirely upon the composition of that particular water. The various compounds have differing disinfecting powers with free residual chlorine as the most active.

The mixture of inorganic and various undetermined organic chlorine compounds causes difficulties when analysing the residual chlorine in testing the hygienic quality of drinking and bathing water. With the orthotolidine method, one of the most commonly used chlorine tests, one can differentiate between free and

combined available chlorine. This method is, however, rather uncertain and is to a high degree dependent upon the manual skill of the analyser. In an examination of 193 American swimming pools it was found that the free and combined available chlorine was measured correctly with the orthotolidine method according to the Standard Methods Instructions in only one of them (Black *et al.* 1970; Orland, 1965). In Sweden, also, from our experience, often only the total available chlorine is measured.

Other analytical methods allow a differentiation between free and combined available chlorine. Some of them can differentiate between the various inorganic chloramines (Nicolson, 1965). However, with none of the methods now known can one determine which organic chloramines are present in the analysed material. These may also have an appreciable disinfecting action (Moore, 1951).

Furthermore, in the same water, differing methods of analysis have been shown to give different values when the free or total available chlorine has been measured (Nicolson, 1965; Katz & Heukelekian, 1959; Rand & Hunter, 1961). When comparative measurements were made at this laboratory during analysis of pure solutions of NaOCl, NH_2Cl and $NHCl_2$, the orthotolidine method showed values which were about 90 % of the values for free or combined available chlorine found with amperometric titration. For the organic chlorine compounds chloramine T and halazone, the orthotolidine method gave only approximately half of the values for total available chlorine found with amperometric titration. When some chlorinated swimming-pool waters containing organic material were analysed, the orthotolidine method gave on the average about half of the values shown by the amperometric method for combined available chlorine, and also in a few cases of free available chlorine. In the orthotolidine method, the total available chlorine is determined by measuring the yellow colour achieved by the oxidation of the orthotolidine reagent at pH 1·3 after 5 min. During that time, many organic chloramines will not yet have reacted with the orthotolidine, so they will not show up in the determination. The coloration continues to rise $\frac{1}{2}$ hr. after the pool water has been analysed. In the amperometric titration of total available chlorine, on the other hand, the chlorine compounds oxidize potassium iodide at pH 4, after which the freed iodine is titrated amperometrically with phenylarsine oxide (Orland, 1965). In the pure solutions of organic chloramines and the swimming-pool waters mentioned above it was apparent that a greater amount of organic chlorine compounds reacted to potassium iodide within a few seconds than to orthotolidine during 5 min.

Consequently, the measuring of residual chlorine which is undertaken at waterworks and public swimming pools, especially when only the total available chlorine is measured, is an uncertain way of determining the various active chlorine compounds contained in the water. Thus, the measurements do not give a relevant indication of the disinfecting properties of the water. In practice, using chemico-physical examinations and bacteriological inspections, it is often decided on the basis of experience how high the total available chlorine level should be in order to achieve bacteriological safety in a certain water. That value will hold true, however, only if the composition of the water is not altered. The procedure

becomes particularly unsatisfactory at public swimming pools, where the number of bathers varies widely and therewith, the quality of the water varies pronouncedly within a relatively short time.

It has been suggested that the measurement of a chlorinated water's redox potential could be used as an alternative way of assessing the disinfectant quality of a water (Frers, 1951; Luck, 1966). Such measurements provide an indication of the effectiveness of the oxidizing agents present. Earlier investigations, however, have shown a poor agreement between the redox potential and the killing of *Entamoeba histolytica* cysts (Chang, 1945), and *Bacillus anthracis* spores (Heicken, 1956). In one investigation a simultaneous measurement of the redox potential and the killing of bacteria was not made (Schmelkes, 1933; Schmelkes & Horning, 1935). Objections raised against the application of the redox potential to chlorine measurements are based on the fact that it is not specific for chlorine and chlorine compounds (Babcock, 1966). On the other hand, in studies on the inactivation of polio virus through the effect of different oxidizing agents, Lund (1963, 1965) reported a proportional relationship between the redox potential and the inactivation of the polio virus both in distilled water and waste water.

The present experiments were performed in order to investigate the usefulness of redox measurements to determine the bacteria-killing capacity of a chlorinated water. If the redox potential in a water could be directly related to the bacteria-killing effect, a simple and readily available method for determining the hygienic quality of a water would be available.

In the investigation various pure, defined chlorine compounds were studied in water and the correlation between active chlorine, redox potential and bactericidal capacity was studied.

During the course of the experiments a report was published by Carlson, Hässelbarth & Mecke (1968). They had investigated the relationship between the redox potential and the rate of the bacterial kill using *E. coli* in chlorinated swimming-pool water and water given a high content of organic material by the addition of gelatin and NaCl. Despite relatively large variations, the rapidity of the reduction of viable bacteria was found to be better correlated to the redox potential than to the residual chlorine, measured with the DPD-ferrous sulphate method (Palin, 1957, 1958). In a long-term experiment, a decrease of the disinfecting effect of the water was reflected in a decreasing redox potential.

The above-mentioned experiments were performed directly upon pool water, the exact composition of which was unknown. Apart from differentiating free and combined available chlorine, no consideration was given to the different chlorine compounds which can be formed in the water. The present experiments with pure chlorine solutions in chlorine-demand-free water elucidate the characteristics of various chlorine compounds in a water solution under standardized conditions.

METHODS AND MATERIALS

A suspension of *E. coli* was put into chlorine-demand-free water, in which various chlorine compounds were dissolved. The number of viable bacteria was determined after 3 min. of contact between chlorine and bacteria. The redox potential and the available chlorine were measured directly before and after the contact period.

The bacterial suspension had as a rule a density of 2×10^5 to 5×10^5 bacteria/ml. Since the redox potential is strongly influenced by pH it was kept at 7·0 in all experiments. The temperature in all experiments was about 22° C.

Portions of 700 ml. buffered chlorine-demand-free water, pH 7·0, were autoclaved in 1 l. Erlenmeyer flasks with a ground-in plug. A concentrated solution of a chlorine compound was added up to a calculated concentration, after the same amount of water had been removed. After mixing, 100 ml. were withdrawn for measuring the redox potential and 200 ml. for measuring the free and combined available chlorine. To the remaining 400 ml. was added a 10 ml. bacterial suspension. A remixing took place and at the end of the 3 min. contact period, 100 + 200 ml. were withdrawn to be measured with regard to redox potential and available chlorine. After exactly 3 min. the reaction between chlorine and bacteria in the remaining 110 ml. in the flask was interrupted with 5 ml. of sterile 0·07 % (w/v) sodium thiosulphate. Serial tenfold dilutions were made and 1 ml. samples of each were mixed with violet red bile agar in Petri dishes. Colonies were counted after 24 hr. at 37° C. Two plates were made from each dilution and the average number of colonies was recorded.

The glassware was acid-washed and stored overnight filled with a strong hypochlorite solution, after which it was carefully rinsed with chlorine-demand-free water.

The chlorine-demand-free water was prepared by adding 150 ml. 0·45 M phosphate buffer pH 7·0, and hypochlorite to a concentration of about 4 mg Cl_2/l. to 5 l. double-distilled water. The water was allowed to stand at least 48 hr., after which it was de-chlorinated with a saturated sodium sulphite solution diluted 1/10 to about 0·1 mg. Cl_2/l. It was then boiled, allowed to cool, poured into flasks and autoclaved. During autoclaving, the last residual chlorine disappeared. The flasks were allowed to stand overnight before they were used in order to get a stabilized oxygen saturation.

Sodium thiosulphate solution. Sterile 5 ml. portions of sodium thiosulphate 0·7 g./l. were used for interrupting the reaction between chlorine and bacteria. $Na_2S_2O_3$ at this concentration does not affect the bacteria.

Bacterial suspension. For all experiments *Escherichia coli* strain B stored in tubes with nutrient agar were used. After incubation in 4 ml. nutrient broth for 4 hr. and an additional 20 hr. growth in 100 ml. nutrient broth, the bacteria were centrifuged, washed 4 times with sterile, chlorine-demand-free buffered water to get as low chlorine demand as possible, suspended in 100 ml. chlorine-demand-free buffered water and diluted 1/10. Between the various tests undertaken during a day, the bacterial suspension was kept in a refrigerator.

Redox measurements. Redox measurements (Radiometer, 1966) were made on a Radiometer pH-meter with a platinum electrode (Radiometer P 101). The course of all of the measurements was followed using a recorder. The measurements were carried out with a calomel electrode as standard, which has a normal potential against the standard hydrogen electrode of +244 mV. at 25° C.

The platinum electrode was rinsed between measurements with chlorine-demand-free water. After each day's use it was cleansed in the manner recommended by Radiometer (1966).

The 100 ml. samples withdrawn were measured with slow magnetic stirring in a 250 ml. beaker of the tall, narrow type. The redox potential reached its maximal value after about 5 min., where it then was kept more or less stable, depending upon which chlorine compound was involved. The maximal value has been noted throughout.

Measurement of available residual chlorine was made on a Wallace and Tiernan amperometric titrator according to Standard Methods Instructions (Orland, 1965).

Chlorine compounds

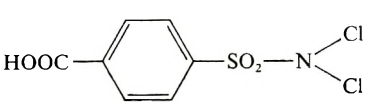
In the experiments, the following chlorine compounds were studied:

Sodium hypochlorite (*NaOCl*). A commercial solution of sodium hypochlorite was used. The compound is dissociated into OCl^- and HOCl in water. At pH 7 about 75 % exists as HOCl and 25 % as OCl^-.

Monochloramine (NH_2Cl). A solution of this was prepared in the following manner. A 0·5 l. sodium hypochlorite solution containing 200 mg. Cl_2/l. was prepared in 0·02 M phosphate buffer, pH 8·5. A solution of ammonium chloride was prepared by dissolving 4·49 g. NH_4Cl in 100 ml. of water; 10 ml. of this was added to 500 ml. of 0·02 M phosphate buffer, pH 8·5. The hypochlorite solution was added, while stirring, to the ammonium chloride solution; the resulting solution contained 1·41 mmol. Cl_2 and 8·39 mmol. NH_4^+ per l. For the experiments, the desired volume of this solution was added to 700 ml. buffered water with pH 7·0. In no case was such a large volume of the monochloramine solution added as would have caused a rise of that pH. The same relationship NH_2Cl-NH_3 was maintained throughout all the experiments. The ammonium concentration influences the redox potential with a maximum of 20 mV. for 0·1 mmol. NH_4^+/l. or 1·2 mg. Cl_2/l. in the experiments. This effect was considered negligible.

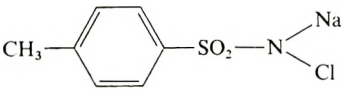
Dichloramine ($NHCl_2$). The dichloramine solution was prepared by adding 1 M-HCl slowly, while stirring, to a monochloramine solution until the pH dropped to 3·5. The decomposition was completed in 24 hr. The solution resulting was volatile.

Halazone (*tablets for water purification*). Halazone is an organic chloramine with the following structural formula:

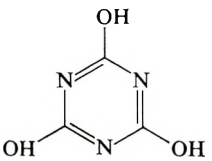


Halazone forms a volatile solution in which free available chlorine can be freed.

Chloramine T. Chloramine T is an organic chloramine with the structural formula:



Cyanuric acid. Cyanuric acid is an organic compound with the structural formula:



It has the capacity for binding one, two or three positive chlorine ions and has come into use as an additive to chlorination in swimming pools. Cyanuric acid in itself does not influence the redox potential of the buffered chlorine-demand-free water or the viability of the bacteria. Combinations with NaOCl and NH_2Cl, added in various amounts to the buffered water containing 50 mg. cyanuric acid/l., were studied in the experiments.

Reduction of bacteria was studied at various concentrations of the different compounds. For the seven different chlorine compounds, a total of 109 bactericidal tests were carried out, divided into 26 different rounds of experiments.

RESULTS

In the experiments with hypochlorite, the concentration of free available chlorine was too low to be measured. This value was then calculated, starting from a stock solution of hypochlorite. In all other experiments, the measured total available chlorine was recorded. In all of these, no free available chlorine could be detected.

When the redox potential was measured in the experiments with hypochlorite, a rather unstable value was obtained. In a few cases the maximum redox potential was reached before it could be recorded. The dichloramine solution also gave slightly unstable potentials, just as did halazone to a certain extent. The redox potentials of the remaining chlorine compounds were stable.

The standard curves of the content of active chlorine of the different chlorine compounds in relation to the redox potential to which they give rise are shown in Fig. 1. NaOCl forms free available chlorine, and the others combined available chlorine. It is evident from the figure that the same amount of available chlorine in the different chlorine compounds gives varying redox potentials. The free available chlorine has a stronger effect upon the redox potential than the combined forms.

The values measured for redox potential and available chlorine in the bactericidal experiments are shown in Table 1 as mean values, calculated from 1–6 values, within each log unit reduction of viable bacteria. The value of the redox potential

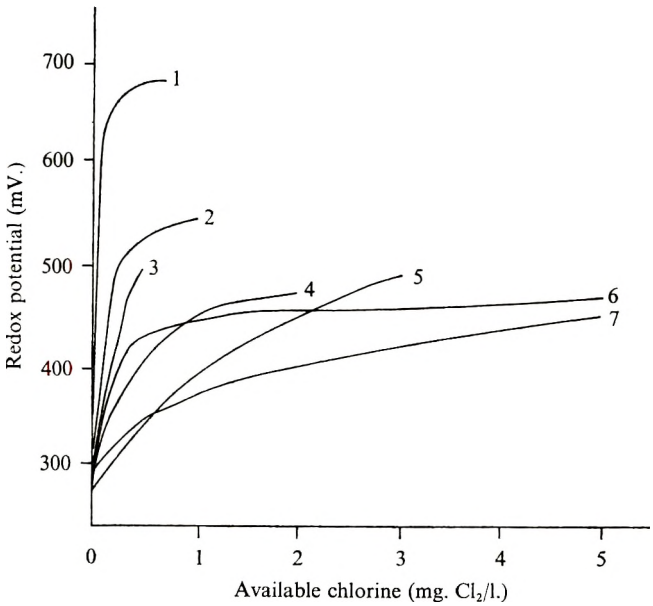


Fig. 1. Relationship between available chlorine and redox potential for different chlorine compounds. 1, Hypochlorite. 2, Dichloramine. 3, Cyanuric acid + hypochlorite. 4, Cyanuric acid + monochloramine. 5, Halazone. 6, Monochloramine. 7, Chloramine T.

before the addition of the bacteria was used. It decreased at an average of 20 mV. during the period of contact between the chlorine and the bacteria, whereas the available chlorine measured did not decrease noticeably. It is seen from the table that, disregarding certain deviations dependent upon the experimental conditions, the reduction of *E. coli* increased as the redox potential and available chlorine for the different chlorine compounds increased.

It is also seen in the table that the chlorine compounds examined give total kill of *E. coli* at a largely varying concentration of free or combined available chlorine. The amount of free available chlorine required was only 1/9 and 1/6 of that required for combined available chlorine in the form of the inorganic chloramines NH_2Cl and $NHCl_2$ respectively. These in their turn are more active than the organic chloramine halazone and much more active than chloramine T. During the 3 min. period cyanuric acid reduces the effect of NH_2Cl and forms a combined residual with hypochlorite with lower disinfective power than pure hypochlorite.

It is also seen in the table that the variation in redox potential level at total bacterial kill is less than the variation in available chlorine for the different compounds. Table 2 shows a calculation of the correlations between the reduction of the number of viable *E. coli* measured in the experiments and the simultaneously measured redox potential and available chlorine for different chlorine compounds.

It is evident from the table that within each chlorine compound both the available chlorine and the redox potential are relatively well correlated to the reduction of bacteria. The correlation for the available chlorine is higher, except in the cases of halazone and cyanuric acid with NH_2Cl.

Table 1. *Percentage survival of* Escherichia coli *within each log unit of reduction compared with available chlorine and redox potential for different chlorine compounds*

(Means from 1–6 values)

Chlorine compound	Survival of *E. coli* (%)	Available chlorine (mg. Cl_2/l.)	Redox potential (mV.)
Sodium hypochlorite NaOCl	100	0	312
	60	0·008	307
	9	0·02	345
	0·9	0·03	330
	0·05	0·04	356
	0·003	0·05	465
	0	> 0·06	> 490
Monochloramine NH_2Cl	100	0	311
	37	0·39	393
	0·9	0·53	370
	0	> 0·53	> 450
Dichloramine $NHCl_2$	100	0	320
	50	0·15	453
	8	0·23	485
	0·04	0·24	503
	0·001	0·18	524
	0	> 0·35	> 530
Halazone	100	0	316
	0·03	0·4	355
	0·007	1·0	455
	0	> 1·0	> 455
Chloramine T	100	0	305
	58	2·3	406
	3	5·3	468
	0·1	5·2	460
	0·005	7·9	480
	0	> 7·9	> 500
Cyanuric acid + NaOCl	100	0	310
	54	0·07	333
	4·0	0·15	364
	0·1	0·15	380
	0·01	0·13	425
	0·005	0·20	449
	0	> 0·34	> 540
Cyanuric acid + NH_2Cl	100	0	311
	28	0·48	403
	0·001	0·69	414
	0	> 0·69	> 465

Considering that the available chlorine present in water actually consists of a mixture of different chlorine compounds, Table 3 shows the correlation between the reduction of number of viable bacteria and the available chlorine and redox potentials for all 109 sets of values, without taking into consideration which chlorine compound was investigated.

The correlation coefficient 0·31 for the relationship between the reduction of *E. coli* and measured combined available chlorine is obtained if the experiments

Table 2. *Correlation coefficient for the relationship between reduction of* Escherichia coli *and simultaneously measured available chlorine and redox potential for different chlorine compounds*

Chlorine compound	Available chlorine (r)	Redox potential before addition of bacteria (r)
NaOCl	0·77	0·56
NH_2Cl	0·98	0·62
$NHCl_2$	0·89	0·86
Halazone	0·67	0·89
Chloramine T	0·97	0·66
Cyanuric acid + NaOCl	0·84	0·63
Cyanuric acid + NH_2Cl	0·63	0·67

Table 3. *The correlation between the reduction of viable* Escherichia coli *and the available chlorine and redox potential computed for all experimental results*

	(r)
Total available chlorine	0·24
Combined available chlorine	0·31
Redox potential	0·50

with free available residual chlorine, hypochlorite, are excluded. The correlation coefficient 0·24 is obtained if all experiments are included in the calculations.

It is clear from the table that a fundamentally higher correlation exists for the redox potential than for the total or combined available chlorine with respect to the reduction of bacteria.

DISCUSSION

In the experiments with combined available chlorine, a little free available chlorine can appear at the same time in certain cases, which then causes the redox potential to rise, without a corresponding increase in the available chlorine measured. In an analogous manner, one finds in experiments with free available chlorine, hypochlorite, a decreasing redox potential if an extremely small chlorine demand appears in the water. These conditions explain the poor relationship sometimes obtained between the available chlorine and the redox potential. In addition, the redox potential sometimes drops after the addition of the bacteria without a corresponding decrease in the measured available chlorine. A possible explanation is that the active chlorine forms compounds with the bacteria or with products excreted from the bacteria, which are still registered as active chlorine when the available chlorine is measured but have a lower redox potential than the chlorine compound used. The other explanation is that, as mentioned above, a small amount of free available chlorine is present which disappears when the bacteria are added.

The different chlorine compounds were found to have largely varying capacities for killing *E. coli*, according to Table 1. This means that a completely misleading result may be obtained when calculating the disinfecting effect of a water, because

the usual methods employed in practice for measuring the available chlorine, do not give any idea of the proportions of the different chlorine compounds.

The values of the redox potential required for total kill according to Table 1 vary for the different chlorine compounds. In water that is unknown and contains a mixture of various chlorine compounds and organic material, one would probably require a higher redox potential than the average value of 500 mV. for bacteriological safety, since one then tries to attain a total bacterial kill that is virtually instantaneous.

In the laboratory experiments only *E. coli* were used as test bacteria. Other bacteria, bacterial spores and viruses can be more resistant against chlorine, and require a higher redox potential for total kill. Lund (1963) reports a redox potential of more than 650 mV. for one log unit reduction of active poliovirus within 30 sec., and 550 mV. for the same reduction within 5 min.

The experiments show that the reduction of *E. coli* is better correlated to the redox potential measurements than to the available chlorine measurements if the values from the different chlorine compounds are compiled (Table 3). If one is uncertain about which chlorine compounds the water contains, the measured redox value thus becomes more reliable than the measured available chlorine value in order to establish the bacteria-killing effect present in the water.

The correlation between reduction of viable *E. coli* and the redox potential found in these experiments is, however, not complete, although the correlation is better than that measured for available chlorine. From the analytical viewpoint the method using measurement of the redox potential is therefore not totally satisfactory for establishing the bacteria-killing capacity of a chlorinated water. Considering the scope and importance of the problem, however, every positive alternative to the accepted methods of analysis must be considered an improvement, especially from the point of view of practical applicability.

A continuously registering redox potential gauge seems to be a good alternative to the present methods commonly used with its few measurements of total available chlorine, especially for public swimming pools with varying bathing loads and water qualities. Field experiments have started in order to assess the significance of this conclusion. Further, the relationship between the redox potential and the reduction of bacteria will be investigated with regard to other halogens, primarily bromine and bromine compounds.

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An epidemic associated with echovirus type 18

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SUMMARY

During the period October 1968 to March 1969 echovirus type 18 was isolated from 83 patients investigated at Fairfield Hospital for Communicable Diseases, Melbourne. The illnesses most commonly associated with these isolations were aseptic meningitis, and fever with rash.

We believe that this is the first report of an epidemic due to echovirus type 18 and the first occasion on which this virus has been shown to produce disease in adults.

INTRODUCTION

Echovirus type 18 (hereafter referred to as echo 18) is an infrequently encountered virus which has rarely been associated with clinical disease.

The prototype strain 'Metcalf' was isolated by Ramos-Alvarez & Sabin (1958) from a rectal swab obtained from an 8-month-old child with diarrhoea. Eichenwald, Ababio, Arky & Hartman (1958) demonstrated infection in 17 infants investigated during an epidemic of diarrhoea in a hospital nursery, by isolation of the virus from faecal specimens and detecting simultaneous antibody rises. Echo 18 has also been isolated from the faeces of a 5-month old child who died after a short febrile illness (Sabin, Krumbiegel & Wigand, 1958), from multiple sites in a 3-week-old child with fever and rash (Medearis & Kramer, 1959) and from the C.S.F. and faeces of a 12-year-old boy with aseptic meningitis (Eckert, Barron & Karzon, 1960). The latter two patients developed rises in homologous antibody titre.

In August 1968 a virus was isolated from the cerebrospinal fluid of a 7-year-old boy with aseptic meningitis admitted to Fairfield Hospital for Communicable Diseases, Melbourne. Preliminary studies suggested that this strain, designated R.B., was an enterovirus but it was not typable by procedures routinely used in this laboratory. Further study showed it to be a variant of echo 18.

From October 1968 to March 1969 strains similar to the R.B. virus were obtained from an additional 83 patients investigated at this hospital.

This communication gives an account of the procedures used in the isolation and identification of these strains.

MATERIALS AND METHODS

Specimens

Throat swabs

Throat swabs were collected into screw-capped bottles containing 2–3 ml. of 'virus transport medium' consisting of serum-free Eagle's Medium (B.M.E.) and antibiotics (penicillin, 100 i.u./ml.; streptomycin, 100 μg./ml.; neomycin, 40 units/ml.; amphotericin B, 20 μg./ml.). On arrival at the laboratory the bottles were agitated on a mechanical shaker for 10 min., the fluid transferred to a centrifuge tube and spun at 1200 rev./min. (400g) for 10 min. and the resulting supernatant fluid inoculated into tissue cultures.

Cerebro-spinal fluid

Cerebro-spinal fluid (C.S.F.) was collected into dry sterile tubes and cultured without treatment. Specimens which were obviously bloodstained were centrifuged at 1200 rev./min. (400g) for 10 min. and the supernatant fluid used for inoculation.

Throat swabs and C.S.F.'s taken at night or on weekends were stored at 4° C. and processed within 48 hr.

Faeces

About 10 g. of faeces were collected into sterile screw-capped jars and stored at −20° C. The specimens were subsequently thawed and 20 % suspensions prepared using transport medium as diluent. After centrifugation at 2000 rev./min. (1400g) for 15 min. the supernatant fluid was collected and re-spun for 20 min. at 10,000 rev./min. (26,000g) in a 'multi-speed head' of an International PR2 refrigerated centrifuge. The resulting supernatant fluid was decanted and its pH adjusted to 7·2–7·4 by the addition of 4 % hydrochloric acid or 5 % sodium bicarbonate.

Serum

Serum samples obtained during the acute and convalescent phases of the patient's illness were stored at −20° C. until required.

Cell cultures

Cell cultures were prepared in screw-capped, flat-bottomed, glass phials (72 × 20 mm.)* and incubated stationary at 36° C. until the growth was almost confluent.

A summary of the cells and culture media used is shown in Table 1.

The diploid human embryonic fibroblasts were established from the lungs of a locally obtained embryo. MEK-3, a heteroploid epithelial cell line, was derived from kidneys of a cynomolgus monkey foetus and has been maintained in this laboratory since 1963.

* Suppliers: Johnsen and Jorgensen, London, England.

Table 1. *Cells and culture media*

Designation	Cell type	Growth medium*		Maintenance medium†	
		Medium	Serum	Medium	Serum
MK	Primary cynomolgus monkey kidney	199‡	2% foetal calf	Eagle's	2% foetal calf
HEL	Diploid human embryonic lung fibroblasts	Eagle's§	5% calf	Eagle's	2% foetal calf
HeLa	Heteroploid human epithelial cells	Eagle's	5% calf	Eagle's	2% foetal calf
HEp-2	Heteroploid human epithelial cells	199	5% calf	Eagle's	2% foetal calf
RK13	Heteroploid rabbit kidney epithelial cells	199	5% calf	199	1% foetal calf
BSC-1	Heteroploid cercopithecus monkey epithelial cells	199	5% calf	Eagle's	2% foetal calf
MEK-3	Heteroploid cynomolgus monkey embryonic kidney	199	5% calf	Eagle's	2% foetal calf

* All contain penicillin 100 i.u./ml., streptomycin 100 μg./ml.
† All contain penicillin 100 i.u./ml., streptomycin 100 μg/ml., and neomycin 40 units/ml.
‡ Obtained from Commonwealth Serum Laboratories, Melbourne, Australia.
§ Basal Medium Eagle's, diploid, obtained from Grand Island Biological Co., Grand Island, New York, U.S.A.

Isolation of viruses

All specimens were inoculated into duplicate screw-capped phials of MK and HeLa tissue. Throat swabs from patients presenting with a rash were also inoculated into HEL, BSC-1 and RK13 tissue in an attempt to isolate rubella virus.

Immediately before inoculation the growth medium was poured off and the inoculum added, together with sufficient maintenance medium to bring the final volume of fluid to 2 ml.

For throat swabs, 0·2 ml. of material was used, whereas each C.S.F. specimen was distributed equally among the phials.

Undiluted faecal extracts were dispensed into HeLa cells (0·2 ml.) and monkey kidney (0·5–0·7 ml.), which were rotated on a roller drum at room temperature for 1 hr. The inoculum was then poured off, the tissues washed once with maintenance medium and a further 2 ml. of this medium added. When cytotoxic faecal extracts were encountered, further cultures were seeded with one-third to one-half the original inoculum and, if the cells were again destroyed, a blind passage was made in a final endeavour to recover a virus.

All cultures were incubated at 34° C. on roller drums rotating at 10 rev./hr. After 3–4 days they were examined using the low-power (× 100) of a Leitz inverted microscope. If a cytopathic effect (C.P.E.) were seen the phials were re-examined daily. In the remaining cultures the medium was decanted, fresh medium added and incubation continued. This procedure was repeated twice weekly for 3 weeks, after which, if no changes had occurred, the cultures were discarded. No blind passages were made unless cell disintegration took place before this time.

Identification of enteroviruses

Cultures showing a C.P.E. were incubated until 50-100% destruction of cells occurred. The culture medium was decanted, centrifuged at 1500 rev./min. (800*g*) for 15 min. and the supernatant tissue culture fluid (T.C.F.) was tested as follows:

Haemagglutination

Human group O erythrocytes washed three times and resuspended in phosphate buffered saline, pH 7·0, were used as a 0·5% suspension. One drop (0·03 ml.) each of undiluted T.C.F. and red cell suspension were mixed in a polystyrene microtitre U plate* with controls in which maintenance medium was substituted for T.C.F. The test was incubated at room temperature (25° C.) and read when the cells in the controls had settled to a compact button (30–60 min.).

Tissue specificity

One drop (0·03 ml.) of T.C.F. was seeded into duplicate phials of MK, MEK-3, HeLa, HEp-2 and HEL cells. The cultures were incubated at 34° C. on a roller drum and examined daily for C.P.E.

Newborn mouse inoculation

A litter of eight newborn mice was inoculated intracerebrally with 0·05 ml. and intraperitoneally with 0·05 ml. of T.C.F. The inoculated animals were observed for 3 weeks. No histopathology or blind passages were carried out.

Serotyping by neutralization

The method used followed that described by Lennette & Schmidt (1969), with the following exceptions:

(1) The test was assayed in the cells showing maximum sensitivity in the tissue specificity test.

(2) The virus–serum mixtures were incubated for 1 hr. at room temperature.

(3) Duplicate cell cultures were each inoculated with 0·1 ml. of virus–serum mixture.

If the tissue specificity of the strain were typical of a particular group of viruses, the antisera thus suggested were used. In all other cases the viruses were tested against pools of high-titre antisera and then against the individual sera of the pool which produced neutralization.

Neutralizing antibody estimations

Neutralizing antibody titrations were performed with serial fourfold dilutions of serum (beginning at 1/4) and 100 TCD50 of virus, controls being set up as for serotyping. Acute and convalescent serum samples were tested simultaneously.

* Cooke Engineering, Alexandria, Va., U.S.A.; catalogue no. 220/24A.

Preparation of rabbit antiserum

The R.B. strain, which had been purified by three terminal dilutions, was cultured in MEK-3 monolayers in 6 oz. flat-sided glass bottles each containing 20 ml. of maintenance medium. When cell destruction was complete, the contents of the bottles were frozen and thawed three times. The material was clarified by centrifugation at 2000 rev./min. (1400*g*) for 15 min. and the virus sedimented by centrifugation at 50,000 rev./min. (150,000*g*) for 3½ hr. in a Spinco Model L 2 ultracentrifuge. The pellet was resuspended in Hanks's Balanced Salt Solution to one-tenth the original volume.

Before inoculation, blood was taken from a 4-month-old rabbit and the serum stored at −20° C. The rabbit was injected intravenously with 1 ml. of the virus concentrate weekly for 5 weeks. A blood sample was obtained 7 days after the last injection and the serum tested for the presence of homologous neutralizing antibody. Because the antibody level was unsatisfactory, the rabbit was boosted on the 9th and 13th weeks and exsanguinated 1 week after the final injection.

Preparation of monodispersed cultures

Monodispersed virus suspensions were prepared by (1) filtration (Schmidt & Lennette, 1970) and (2) deoxycholate treatment (Gwaltney & Calhoun, 1970).

Physical and chemical properties

Acid lability, stability to ether and heat, nucleic acid determination

The methods followed those described by Schieble, Fox & Lennette (1967). However, in the determination of nucleic acid, *Herpes hominis* Type 1 was used as the DNA-containing virus, and 5-iodo-deoxyuridine was substituted for 5-bromo-deoxyuridine.

Antisera to prototype viruses

The following enterovirus antisera were used:

Rabbit antisera (Fairfield Hospital, Melbourne): Echo 1–9, 11–33, Coxsackie A 7, A 9, A 21, B 1–B 6.

Monkey antisera (Commonwealth Serum Laboratories, Melbourne): Polio 1, 2, 3.

Monkey antisera (National Institutes of Health, U.S.A.): Echo 1–9, 11–31; Coxsackie A 1–A 18, A 20, A 21, A 22, A 24; B 1–B 6; Polio 1, 2, 3.

Viruses

The prototype viruses, obtained from the National Institutes of Health (U.S.A.), used in neutralization tests, were:

Echo 1–9, 11–33, except Echo 23 (local strain used).

Coxsackie B 1–B 6.

Coxsackie A 2, A 3, A 7, A 9, A 10, A 12–A 18, A 20, A 20 A, A 20 B, A 21, A 24.

Polio 1, 2, 3.

The epidemic strains used for cross-reactions and antibody studies and their origin are shown in Table 2.

Table 2. *Strains used for cross-reactions and antibody studies*

Patient	Age	Sex	Source	Diagnosis
R.B.	7	M	C.S.F.	Aseptic meningitis
K.H.	4	F	C.S.F.	Aseptic meningitis, rubelliform rash
A.M.	2	M	T.S.	Pharyngitis, rubelliform rash
J.G.	28	M	T.S.	Aseptic meningitis
M.H.	28	F	T.S.	Atypical pneumonia, pharyngitis, rubelliform rash
T.F.	2	F	T.S.	Rubelliform rash

T.S. = Throat swab.

Table 3. *Age distribution of patients from whom echovirus 18 was grown*

	Age (years)						
	0–4	5–9	10–14	15–19	20–29	30–39	⩾ 40
No. of patients	19	25	13	8	9	9	0

Table 4. *Isolation rate of echo 18 from various specimens*

Illness	T.S.	C.S.F.	F.
Aseptic meningitis	23/65*	29/52	36/43
Others	15/18	—	4/4

* Numerator = number of strains isolated; denominator = number of specimens tested.

RESULTS

Echo 18 epidemic

In the epidemic period October 1968 to March 1969, echo 18 was isolated from 83 patients.

The age distribution of these patients is shown in Table 3.

Sixty-five patients (78%) had aseptic meningitis with or without exanthem, while in 15 the major clinical feature was a rubelliform rash. The other three patients had gastroenteritis, bronchitis and polymyositis respectively.

During the outbreak 107 strains of echo 18 were isolated from 83 patients, primary isolation occurred in MK on 106 occasions and in BSC-1 in one.

Throat swabs and C.S.F. samples were obtained from the 65 patients with aseptic meningitis soon after their admission to hospital. Echo 18 was isolated from 23 (35%) of the throat swabs and 29 (56%) of the 52 C.S.F.'s available for virus studies (see Table 4). In addition, faecal specimens were obtained from 43 of this group usually within 5 days of admission. The virus was recovered from 36 (84%).

Throat swabs were submitted from all 18 patients with diseases other than aseptic meningitis. Echo 18 was isolated from 15 (84%). In one patient both echo 18 and rubella virus were isolated.

Faecal specimens were obtained from only four of this group and the virus was isolated on each occasion.

Table 5. *Results of cross-neutralization tests*

Virus	Antiserum to echo 18		Antiserum to R.B.
	Rabbit*	Horse†	Rabbit
Echo 18 (Metcalf)	**3200**‡	**3200**	400
R.B.	200	50	**200**

* Prepared at Fairfield Hospital against the Metcalf strain.
† W.H.O. reference antiserum. ‡ Reciprocal of antibody titre.

Characterization of the R.B. *virus*

Isolation

The first strain (R.B.) was isolated in primary MK cell culture. A typical enterovirus C.P.E. appeared after 7 days incubation and progressed to 75% destruction of the cell sheet in 10 days. When passaged, the C.P.E. in MEK-3 resembled that seen in primary MK but was more rapid and complete, while HEL showed a fine granular disintegration which was slow to appear and rarely proceeded to completion. No C.P.E. was seen in HeLa or HEp-2 cells. Tissue culture fluid from MEK-3 monolayers had infectivity titres in the order of $10^{5\cdot0}/0{\cdot}1$ ml., whereas those in MK and HEL were 10- to 100-fold lower.

Other properties

The virus was stable at pH 3, resistant to ether and heating (50° C. for 1 hr.), and was unaffected by treatment with 5-iodo-deoxyuridine. It did not agglutinate human erythrocytes and was not pathogenic in newborn mice.

Serology

The R.B. virus was not neutralized by any of the rabbit or monkey prototype enterovirus antisera.

A rabbit antiserum prepared against this virus had an homologous titre of only 1/200. Eight units of this serum were used in neutralization tests against all the prototype enteroviruses which will grow in tissue culture and echo 18 alone was neutralized.

Cross-neutralization tests using R.B. and the prototype echo 18 (Metcalf) and their homologous sera are shown in Table 5.

The rabbit serum prepared against R.B. neutralized both viruses to similar titres while the two prototype antisera required 16–64 antibody units to neutralize R.B. virus.

Variation in strains

Tissue specificity

In contrast to the prototype, most strains grew more readily in MEK-3 than in MK or HEL. Considerable variation was observed in the tissue specificity of the strains isolated during the epidemic. The tissue specificity of 'Metcalf' and four epidemic strains is shown in Table 6.

Table 6. *Tissue specificity of some selected echo 18 strains*

Virus	Tissue		
	MK	MEK	HEL
Echo 18 (Metcalf)	+ + + + 6*	+ + 7	+ + + + 3
R.B.	+ 7	+ + + + 7	+ + 7
K.H.	+ 7	+ 7	+ 7
A.M.	+ + + + 7	+ + + + 5	+ + 7
J.G.	+ + + + 5	+ + + + 4	+ + + + 2

* Day on which C.P.E. recorded. Degree of C.P.E.: + + + +, 100%; + + +, 75%; + +, 50%; +, 25%.

Table 7. *Neutralization tests with various antisera*

Virus	Antiserum to echo 18		Antiserum to R.B. Rabbit
	Rabbit*	Horse†	
Echo 18 (Metcalf)	**3200**‡	**3200**	400
R.B.	200	50	**200**
K.H.	800	800	3200
A.M.	—	50	200
J.G.	6400	6400	6400

* Prepared at Fairfield Hospital against the Metcalf strain.
† W.H.O. Reference antiserum. ‡ Reciprocal of antibody titre.

Table 8. *Reactions with patients' sera*

Patient	Serum specimen days after onset of rash	Neutralizing antibody to virus				
		T.F.	R.B.	Metcalf	J.G.	K.H.
T.F.	1	< 4	< 4	8	8	< 4
	4	< 4	< 4	256	64	32
M.H.	1	< 4	< 4	< 4	< 4	< 4
	8	< 4	< 4	128	2000	2000

Neither the prototype virus nor any of the epidemic strains produced C.P.E. in HeLa or HEp-2 cells.

Sensitivity to antisera

Strains which showed differences in tissue specificity also varied in their reactions in neutralization tests with antisera made to the prototype and R.B. viruses.

All strains were neutralized by anti-R.B. serum at its homologous titre. With the prototype antiserum some isolates reacted like the prototype virus, while others required up to 64 units to produce neutralization.

Patients' sera

Strain variation was also reflected in the differences in the patients' serological responses. Paired specimens obtained from two patients (T.F., M.H.) were tested against the prototype virus and four epidemic strains (Table 8).

Sera from both patients failed to neutralize strains T.F. and R.B. but there were significant rises in titre to two other epidemic strains (J.G and K.H.) and to the prototype strain.

Removal of viral aggregates by filtration and deoxycholate treatment

To determine whether the low titres of various sera to the R.B. virus were due to aggregation of the virus, the prototype echo 18 and R.B. were (1) filtered and (2) treated with deoxycholate and the neutralization tests repeated. In neither case was there an increase in antibody titres.

DISCUSSION

Although there have been scattered reports of the isolation of echo 18, no major outbreaks attributable to this virus appear to have been documented.

Before the recent epidemic the only strains of echo 18 which we have encountered were obtained from a group of apparently healthy children surveyed during a study of the incidence of viral infections in childhood (unpublished data). From 1958–64 specimens were obtained at least once a month from a group of children at a Melbourne creche (average attendance 50). In 1958 echo 18 was isolated from 13 children, in 1961 from 2 and in 1964 from 13, but no association with illness was apparent. All strains were recovered during the months of August to November.

The recent epidemic in Melbourne extended over a period of 6 months, October 1968 to March 1969, and echo 18 was isolated from 83 patients investigated at this hospital. Although the majority of strains were from children, 18 were from patients between the ages of 20 and 40 years. This appears to be the first occasion that the virus has been implicated in an epidemic in adults.

Sixty-five patients were admitted to hospital with aseptic meningitis. Echo 18 was recovered from the C.S.F. of 29 and from the throat or faeces of the remainder. As the virus was recovered from the C.S.F. it is reasonable to suggest a causal relationship between the isolations and the disease.

Echo 18 was isolated from 15 patients with a febrile illness and rubelliform rash, and the epidemiology is suggestive of an association with the illness.

In patients with aseptic meningitis, echo 18 was isolated more frequently from faecal specimens than from throat swabs or C.S.F.'s. Similar findings have been reported in epidemics due to echo 4 (Karzon *et al.* 1961; Ishii, Matsunaga, Onishi & Kono, 1968), echo 6 (Winkelstein, Karzon, Barron & Hayner, 1957), and echo 30 (Torphy, Ray, Thompson & Fox, 1970).

Identification of epidemic strains was difficult because most could not be neutralized by 20 units of antiserum to the prototype virus. Subsequent tests showed that these strains could be neutralized by a larger dose (e.g. 64 units) of the antiserum. Whilst 4 units of antiserum is sufficient for typing most strains, Kamitsuka, Soergel & Wenner (1961) have shown that some require 20–200 units and Wenner (1962) has suggested routinely using 50–100 units for the identification of enteroviruses.

Strain variation among the epidemic strains was shown by the variation in titre of neutralization tests performed with sera from patients and hyperimmune animals. Similar strain variation during an epidemic due to echo 6 was noted by Karzon, Pollock & Barron (1959).

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Colonization resistance of the digestive tract and the spread of bacteria to the lymphatic organs in mice

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SUMMARY

After oral contamination of conventional mice with high doses of *Escherichia coli*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* the contaminant was recovered in abnormally high concentrations from the duodenum and caecum during the first few days. In this initial colonization phase, evidence of spread was obtained by culturing the cervical and mesenteric lymph nodes and spleen. Longer after contamination the intestinal concentration decreased to normal and spread stopped. In orally antibiotic-treated mice, the situation seen during the initial colonization phase in conventional mice occurred after a much lower oral contamination dose and persisted during the entire observation period of 2 weeks.

INTRODUCTION

In a previous paper (van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees, 1971) experiments were described that indicated that the colonization resistance (CR) of the digestive tract is correlated with the presence of several anaerobic species of the intestinal flora in mice. The CR of the digestive tract for a foreign bacterial species was defined as the logarithm of that oral dose of bacteria that colonized the digestive tract for longer than 2 weeks in 50 % of the animals. For an *Escherichia coli* strain the CR was found to be 7, whereas it was above 9 for a *Klebsiella pneumoniae* and a *Pseudomona aeruginosa* strain. During and shortly after oral antibiotics were given, a rapid drop of the CR was found to extremely low values.

In the present study the colonization of the digestive tract is investigated as well as the spread of the contaminant into the lymphatic organs, i.e. the cervical and mesenteric lymph nodes and the spleen.

This study was performed because differences in survival time were found between contaminated conventional and antibiotic decontaminated mice after lethal irradiation. After oral contamination of irradiated mice with various Enterobacteriaceae species, bacteraemia due to the contaminant was found in 100 % of the decontaminated animals. In the conventional groups, bacteraemia due to the contaminant was dose-dependent and varied in frequency. Depending

on the interval between irradiation and contamination, this frequency varied between 40 and 75 %. These results suggested a difference in colonization pattern between conventional and decontaminated mice after experimental contamination.

MATERIALS AND METHODS

Mice

Conventional female ND2 mice and mice of the same stock treated with oral antibiotics were used. All animals were between 10 and 16 weeks of age. Housing, antibiotic decontamination and isolation procedure were identical with those described in a previous paper (van der Waaij *et al.* 1971).

Contamination

The suspensions for oral contamination were prepared in the same way as described before (van der Waaij *et al.* 1971). The streptomycin resistant (SR) strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were the same as used in our previous publication (van der Waaij *et al.* 1971). In the conventional mice, contamination was performed with three different doses, namely 10^5, 10^7 and 10^9; the same doses as were used in the above-mentioned study. In the antibiotic-treated group of mice, however, only one dose of 10^5 cells was used.

Culturing

To determine the concentration of the contaminant in various parts of the digestive tract at various intervals after contamination, mice were killed in groups of 10 at days 1, 2, 3, 4, 7, 10 and 14. Immediately after death 0·1 g. samples of the contents of the duodenum and the caecum were taken and suspended in 0·9 ml. of brain–heart infusion broth (BHI) (DIFCO) to which 10 mg. of streptomycin per ml. was added to prevent growth of endogenous Enterobacteriaceae species in the conventional group. Subsequently the suspensions were tenfold serially diluted with 0·05 ml. diluting loops in the same medium. At autopsy also a throat swab was taken, and the cervical and mesenteric lymph nodes as well as the spleen were removed for culturing in streptomycin BHI-broth under strict aseptic conditions. Since previous experience had indicated that grinding of the organs gave the same results in culture as were obtained by cutting the organs in small pieces, the latter technique was applied in this investigation. All cultures were incubated at 37° C. and scored as negative when no growth was observed at the fourth day. Positive cultures were subinoculated on Endo-agar for subsequent identification.

RESULTS AND EXPERIMENTS

The colonization of the digestive tract after experimental oral contamination was investigated by culturing throat swabs and by determining the concentration of the contaminant in the duodenum and the caecum. Other parts of the digestive tract were not sampled in this study because in previous experiments it was found

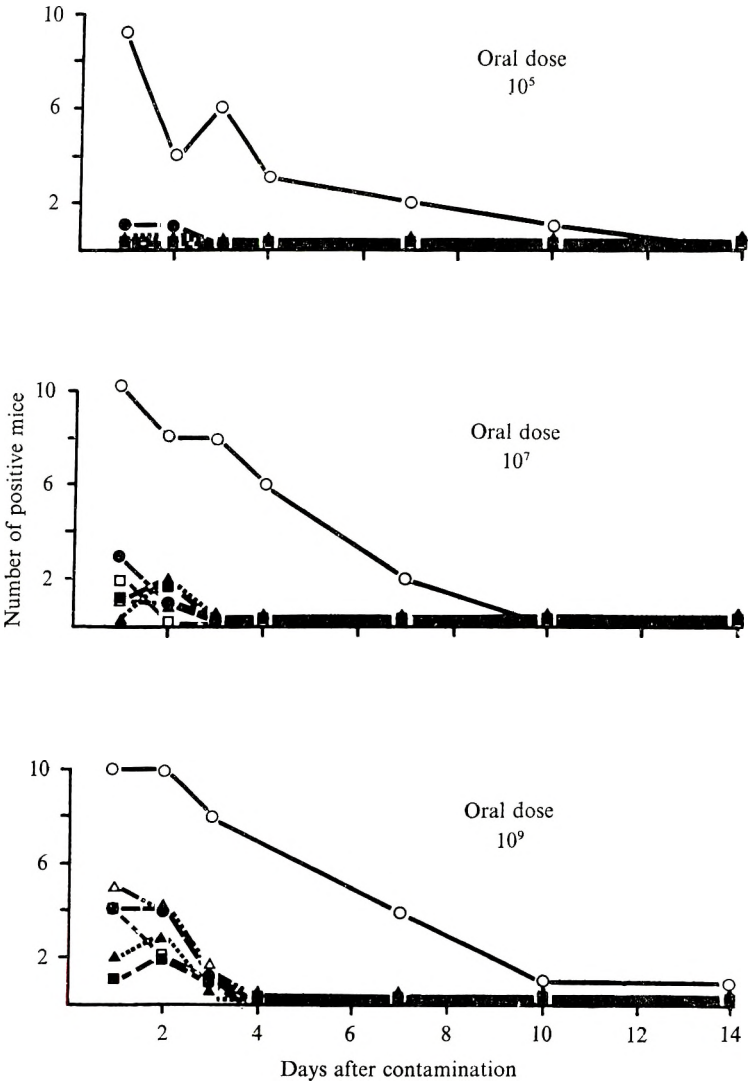

Fig. 1. Conventional mice contaminated with SR-*Escherichia coli*. ○—○, Throat swabs; ■– – –■, cervical lymph nodes; ●– – –●, duodenum concentration > 10^2/g. contents; △–·–·–△, mesenteric lymph nodes; □–·–·–□, caecum concentrating > 10^6/g. contents; ▲· · · ·▲, spleen.

that these three parts of the digestive tract were representative of the colonization pattern in other parts (the stomach, the jejunum, the ileum and the colon). The spread of the strain used for contamination into the regional lymph nodes, i.e. the cervical and the mesenteric lymph nodes, and the spleen, was investigated by culturing these organs in broth.

The results of this study in conventional mice show that abnormally high concentrations of the contaminant in the digestive tract (> 10^2/g. duodenum contents and > 10^6/g. caecal contents) are seen the first 3–4 days following oral doses at the level of CR doses and at higher doses (Figs. 1–3). The CR for the *E. coli*, *Klebsiella* and *Pseudomonas* strains used for contamination were respec-

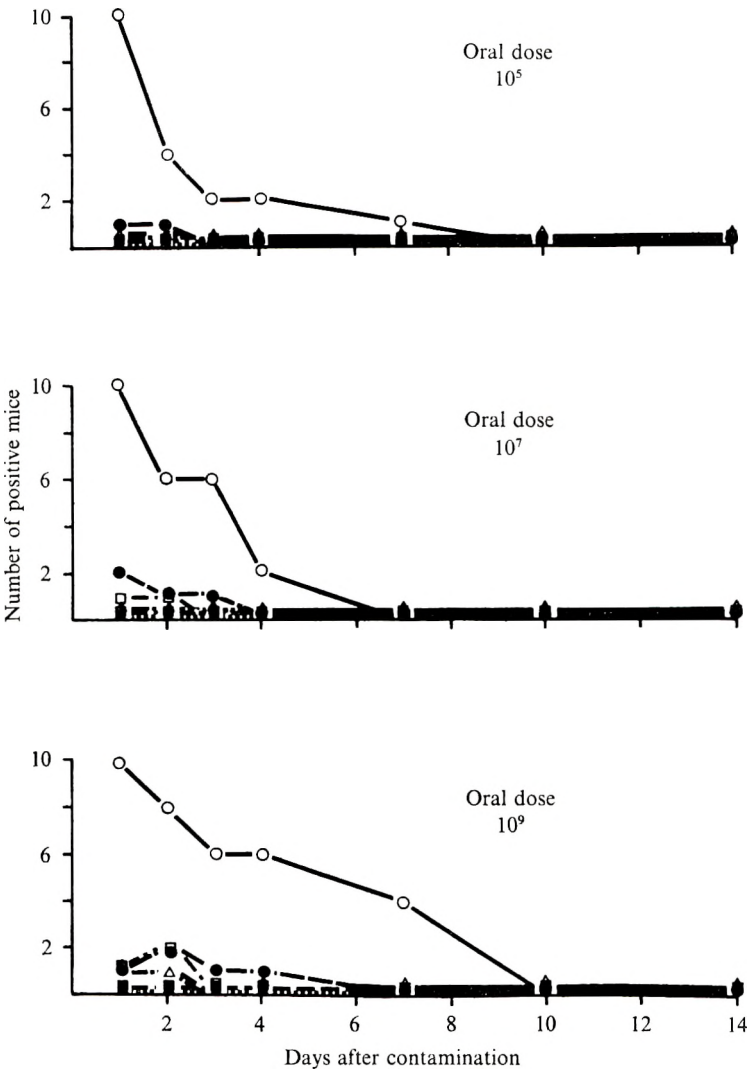

Fig. 2. Conventional mice contaminated with SR-*Klebsiella pneumoniae*. ○—○, Throat swabs; ■– – –■, cervical lymph nodes; ●– – –●, duodenum concentration > 10^2/g. contents; △–··–·· △, mesenteric lymph nodes; □–·–·□, caecum concentration > 10^6/g. contents; ▲· · · ·▲, spleen.

tively 7, 10 and 11. More or less parallel with the 'abnormal' colonization pattern of increased concentration of the contaminant in the digestive tract, positive cultures were obtained from the lymph nodes and spleen of a number of animals. This was most obvious after contamination with SR-*E. coli*, the micro-organism with the lowest CR value (seven). Both other strains, SR-*Pseudomonas* and SR-*Klebsiella*, had a higher CR of above 9. Only after contamination with SR-*E. coli* were abnormally high concentrations and positive cultures of the lymphatic organs found during the first 3 days after contamination. Observations made in mice that were killed later after oral contamination revealed negative cultures of the

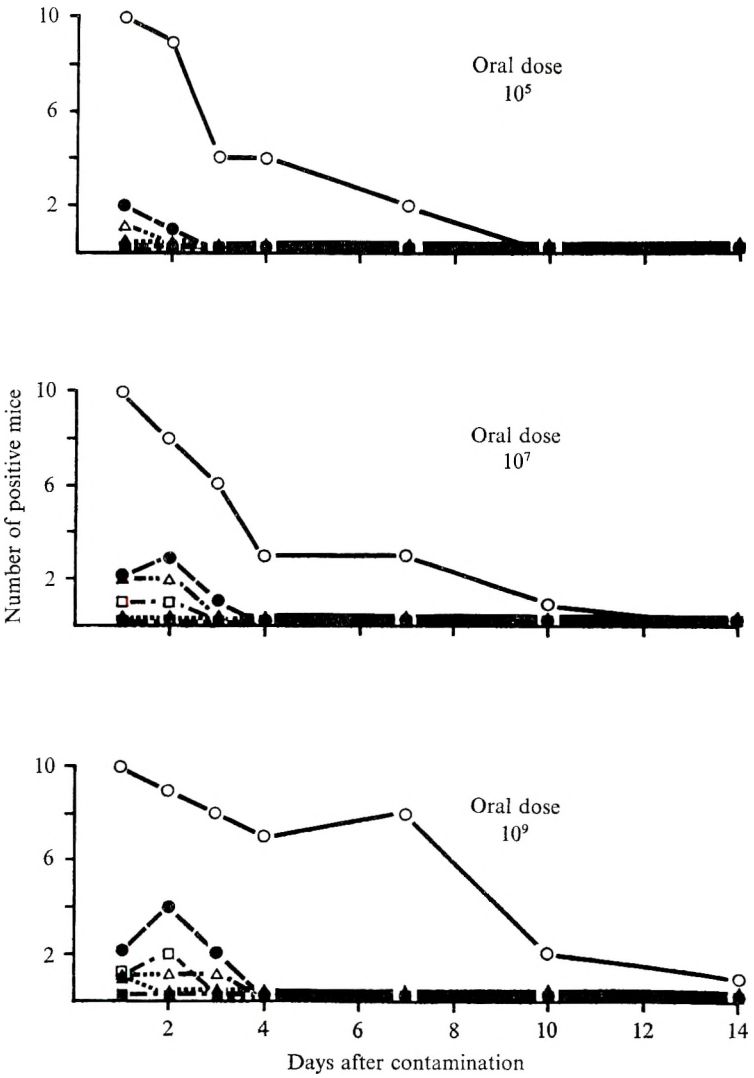

Fig. 3. Conventional mice contaminated with SR-*Pseudomonas aeruginosa*. ○—○, Throat swabs; ■- - -■, cervical lymph nodes; ●- - -●, duodenum concentration $> 10^2$/g. contents; △-·-·-△, mesenteric lymph nodes; □-·-·-□, caecum concentration $> 10^6$/g. contents; ▲····▲, spleen.

lymphatic organs. In the first days after contamination a high percentage of animals had positive throat swabs. This, however, decreased rapidly.

In decontaminated mice abnormally high concentration of the contaminant and frequent positive lymphatic organ cultures were seen at all intervals after contamination with 10^5 cells (Fig. 4). This was most evident in the *E. coli* and in the *Kl. pneumoniae* contaminated mice. After contamination with *Ps. aeruginosa*, increased concentrations in the intestines and positive lymphatic organ cultures were found in a somewhat smaller percentage of the animals. Only the throat swabs remained positive in all decontaminated animals at all intervals after contamination.

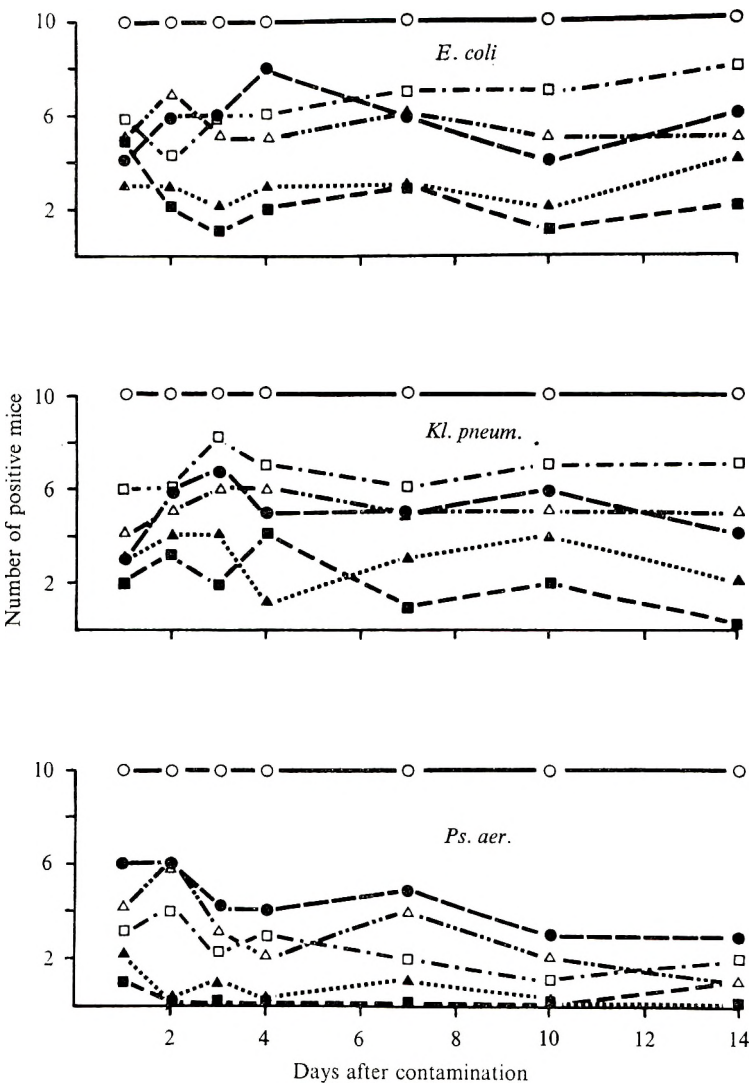

Fig. 4. Orally antibiotic-treated mice contaminated with 10^5 bacteria of three different species. ○—○, Throat swabs; ■- - -■, cervical lymph nodes; ●- - -●, duodenum concentration > 10^2/g. contents; △-·-·-·△, mesenteric lymph nodes; □-·-·□, caecum concentration > 10^6/g. contents; ▲····▲, spleen.

DISCUSSION

The present investigation has shown that in conventional mice abnormal colonization of the digestive tract (sometimes correlated with 'positive' lymph nodes and spleen) is only seen during the first 3–4 days following high oral doses with potentially pathogenic (p.p.) Gram-negative species. Abrams & Bishop (1966) have described a somewhat similar result after intragastric or intraduodenal challenge with *Salmonella typhimurium*.

After this initial colonization phase the situation becomes normal. Cultures of lymphatic organs were found negative in all animals and the concentrations in

which the contaminant was found in the intestines had also decreased to normal values. After the lower contamination doses, in several mice the contaminant disappeared completely.

In mice whose intestinal flora had been greatly reduced by oral antibiotics the situation described above for the initial colonization phase persisted for much longer. Abnormal colonization and evidence of invasion was found during the entire period of suppression of the CR-responsible part of the intestinal flora (Dubos, Schaedler & Stephens, 1963; Savage & Dubos, 1968; van der Waaij *et al.* 1971) by oral antibiotic treatment. A comparable situation is also seen in monocontaminated germ-free mice, as has been described by Schaedler *et al.* (1965). Bonhoff & Miller (1962) described a similar difference in response between conventional and streptomycin-treated mice. The conventional animals required over 5×10^3 *Salmonella enteritidis* cells by stomach tube to make 50 % of the animals positive for some time. With lower doses fewer animals, and with higher doses more animals became positive. Invasion of the spleen was found to occur more frequently after the higher doses and was particularly seen in animals in which the salmonella concentration in the faeces was above 10^2 per faecal pellet. In the streptomycin-treated groups, doses as low as 10 or less made 50 % positive.

The mechanism responsible for the abnormal situation in 'initial colonization phase' seems to depend on an intact microflora. In conventional mice the CR mechanism is presumably temporarily overwhelmed by oral doses at or above the CR determining doses. When the CR is very low (as is seen in germ-free and orally antibiotic treated mice), it evidently cannot any longer control the colonization of the digestive tract, and is overwhelmed even after low oral contamination doses. Because invasion of the lymph nodes and spleen has only been seen in association with high intestinal concentration of the contaminant (both p.p. and pathogenic) we can assume that, in case an abnormal colonization pattern is seen, with positive throat swabs and high concentrations of the same species in the faeces, the mesenteric lymph nodes and the spleen are from time to time invaded by that particular species.

In conclusion we could state that the CR mechanism not only controls the microflora in the digestive tract but also directly or indirectly prevents spread of the endogenous p.p. species. The practical consequences of these observations are being investigated in irradiation experiments. Animals with a good CR due to our CRF flora (van der Waaij *et al.* 1971), which are contaminated and colonized with one p.p. bacterial species, may be less exposed to this p.p. species after irradiation than monocontaminated previously germ-free or antibiotic-decontaminated mice (van der Waaij & Sturm, 1968). These antibiotic-decontaminated mice have much greater numbers of the p.p. strain in their intestines than the CRF group. Also, the spread of infection in monocontaminated mice may be a disadvantage after irradiation or similar treatment has decreased the resistance to infection. Evidence has been obtained that this is the case since, particularly in experiments with the more 'pathogenic' p.p. species, the incidence of infection is much higher and the onset after irradiation earlier in monocontaminated mice than in CRF animals associated with that particular p.p. species (van der Waaij, unpublished data).

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Specificity of early protective responses induced by pseudomonas vaccines

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SUMMARY

A single inoculation of pseudomonas vaccine protected mice against a 1 LD100 challenge by nine different serotypes of *Pseudomonas aeruginosa*, 4 days after vaccination. Three inoculations of the same vaccine, given on consecutive days, protected mice for a longer time, against challenge by a wide range of *Ps. aeruginosa* serotypes, than the single inoculation of vaccine. The amount of vaccine inoculated only marginally influenced the non-specific response. F1 vaccines from eight serotypes of *Ps. aeruginosa* each induced protection against a different range of serotypes.

INTRODUCTION

Failure to protect patients with extensive burns against *Pseudomonas aeruginosa* can result in severe illness, septicaemia and death (Tumbusch *et al.* 1961; Kefalides *et al.* 1964; Jones, Jackson & Lowbury, 1966). Topical chemoprophylaxis has been found effective in preventing colonization of burns, thereby reducing the chance of invasive septicaemia (Lindberg *et al.* 1965; Lowbury & Jackson, 1970), but occasionally fatal infections occur even when the most effective topical chemoprophylaxis has been used (Cason & Lowbury, 1968; Alexander, Fisher, MacMillan & Altemeier, 1969).

Research into alternative methods of preventing invasive *Ps. aeruginosa* septicaemia has shown the value of active immunization (Feller, 1966; Markley & Smallman, 1968; Alexander *et al.* 1969; Jones, 1971). Recent experiments showed that burned mice can be protected against lethal *Ps. aeruginosa* infection by a single injection of vaccine administered 24–48 hr. before infection (Jones, 1971). In the present study, an attempt has been made to determine the degree and specificity of early protective responses induced in mice by a single injection and by three injections on consecutive days of pseudomonas P14F1 vaccine. Experiments were made to test how the size of dose of this vaccine affected the specificity, duration and degree of the protective response. Variations in the specificity of protective responses of mice given single injections of F1 vaccines, extracted from different strains of *Ps. aeruginosa*, are also reported.

MATERIALS AND METHODS

Strains of Ps. aeruginosa *used*

Serotypes 2A, 2A 2B 5C, 3, 5C, 5D, 8, 10, 11, NT (non-typable) and 2/5 (B4) were isolated from patients with burns. Serotypes 1, 6A, 6B, 6C and 9 were isolated from other hospital sources, including tracheostomy sites. Serotypes 2AB and 14 were obtained from Dr M. T. Parker who kindly typed all strains of *Ps. aeruginosa* used in these experiments by serological and phage typing methods.

Vaccines

Pseudomonas P14F1 vaccine was extracted from a culture filtrate of *Ps. aeruginosa* P14, by gel filtration using methods described by Carney & Jones (1968). The same method of extraction was used to obtain F1 vaccines from 1 l. culture filtrates of serotypes 2AB, 3, 5C, 8, 10, NT and strain B4. All vaccines were lyophilized; before injection into mice they were dissolved in physiological saline and sterilized by filtration through a GS Millipore membrane (pore size 0·2 μ).

The vaccines were injected intraperitoneally in 1·0 ml. volumes of saline into 25 g. male Schofield albino mice, by methods described by Jones (1971). The size of dose and frequency of injection of the vaccines is described below.

Infection

In all experiments mice were infected intraperitoneally by injection of 1·0 ml. of a saline suspension of *Ps. aeruginosa*. Groups of mice were challenged with 1 LD100 of *Ps. aeruginosa* at various times after vaccination. Fifteen serotypes of *Ps. aeruginosa* were used for challenging vaccinated mice; the LD100 for these serotypes was determined by i.p. injection into groups of three unvaccinated mice of ten-fold dilutions of saline suspensions ranging from $2{\cdot}1 \times 10^9$ bacteria to $2{\cdot}1 \times 10^6$ bacteria. The numbers of bacteria in the suspensions were estimated from Brown's opacity tubes (Wellcome). Once a dilution of bacterial suspension which killed all the mice had been determined, the numbers of bacteria were reduced by units of the Brown's scale until the smallest number which killed all three mice had been found. Table 1 shows the LD100 of 15 serotypes of *Ps. aeruginosa* for mice determined by this method.

Mice were observed for 48 hr. after challenge, as it was found that mice which survived for this time seldom died later.

In each experiment a control group of three unvaccinated mice received the challenge dose intraperitoneally to confirm its 100% lethality.

Experimental procedures

Specificity of protective responses after a single injection of P14F1 vaccine

Groups of 45 mice were given either 1·0 or 0·01 mg./kg. mouse weight of P14F1 vaccine intraperitoneally. After vaccination, groups of three mice from each of the two groups of vaccinated mice and from a control group of unvaccinated mice were infected intraperitoneally with 1 LD100 of each of 15 different serological types of *Ps. aeruginosa*.

Table 1. *LD*100* *of* 15 *serotypes of* Ps. aeruginosa *for mice*

Serotype of challenge strain of *Ps. aeruginosa*	No. of bacteria injected intraperitoneally into mice
1	$2{\cdot}1 \times 10^8$
2A	$6{\cdot}3 \times 10^8$
2A 2B 5C	$2{\cdot}1 \times 10^8$
3	$2{\cdot}1 \times 10^8$
5C	$4{\cdot}2 \times 10^8$
5D	$2{\cdot}1 \times 10^8$
6A	$6{\cdot}3 \times 10^7$
6B	$6{\cdot}3 \times 10^8$
6C	$6{\cdot}3 \times 10^8$
8	$4{\cdot}2 \times 10^8$
9	$8{\cdot}4 \times 10^8$
10	$4{\cdot}2 \times 10^8$
11	$3{\cdot}2 \times 10^8$
14	$6{\cdot}3 \times 10^7$
NT	$2{\cdot}1 \times 10^9$

* Estimated from Brown's opacity tubes.

Different groups of vaccinated mice were challenged 2, 4, 7 and 14 days after vaccination to see if the protective responses of mice following a single injection of pseudomonas P14F1 vaccine varied at different times after vaccination.

*Investigation of the specificity and degree of early protective responses of mice vaccinated with different amounts of pseudomonas F*1 *vaccine*

Groups of 45 mice were vaccinated with a single injection of 1·0, 0·1 or 0·01 mg./kg. mouse weight of pseudomonas P14F1 vaccine. Four days after vaccination, a time determined by the previous experiment when maximum non-specific protective responses were found, three mice from each of the three groups of vaccinated mice and from a group of unvaccinated control mice were challenged with 1, 5 or 10 LD 100 of 15 different serological types of *Ps. aeruginosa*.

*Specificity of protective responses of mice after a triple injection of pseudomonas P*14*F*1 *vaccine*

Groups of 45 mice were inoculated intraperitoneally on each of three consecutive days with 0·1 mg./kg. mouse weight of pseudomonas P14F1 vaccine. On days 2, 4, 7 and 14 after the last injection of vaccine, groups of three mice were challenged with 1 LD 100 of each of 15 different serological types of *Ps. aeruginosa*.

Specificity of protective responses of mice after single injections of vaccines from different strains of Ps. aeruginosa

Pilot studies, in which groups of mice were given single injections of 1·0, 0·1, 0·01 and 0·001 mg./kg. mouse weight of vaccines made from *Ps. aeruginosa* serotypes 2AB, 3, 5C, 8, 10, B4 and NT, 4 days before 1 LD 100 homologous challenge, showed that all vaccines except NTF1 would protect mice against homologous

Table 2. *Specificity of protective responses in mice after a single injection of pseudomonas vaccine P14F1*

Serotype of challenge strain	Day of challenge after vaccination: 2, Vaccine dose (mg./kg.) 1·0	2, 0·01	4, 1·0	4, 0·01	7, 1·0	7, 0·01	14, 1·0	14, 0·01
1	+	+	+	+	−	−	−	−
2A	−	−	−	−	−	−	−	−
2A 2B 5C	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−
5C	−	−	−	−	−	−	−	−
5D	−	−	+	−	−	−	−	−
6A	−	−	+	+	+	+	+	+
6B	+	+	+	+	+	+	+	+
6C	+	+	+	+	−	−	−	−
8	−	−	+	−	−	−	−	−
9	+	−	+	+	−	−	−	−
10	−	−	−	−	−	−	−	−
11	−	−	+	−	−	−	−	−
14	+	+	+	+	+	+	+	+
NT	−	−	−	−	−	−	−	−

+ = all mice survived; − = one or more mice died.

challenge at a dose of 0·1 mg./kg. mouse weight. A dose of 1·0 mg./kg. mouse weight of NTF1 vaccine was required to protect against the homologous strain.

Groups of 45 mice were vaccinated with 0·1 mg./kg. mouse weight of pseudomonas 2AB, 3, 5C, 8, 10, 14 and B4F1 vaccines respectively and a further group of 45 mice was vaccinated with 1·0 mg./kg. mouse weight of NTF1 vaccine.

Four days after vaccination groups of three mice, from each of the eight groups of vaccinated mice, were challenged with 1 LD100 of each of the 15 different serological types of *Ps. aeruginosa* respectively. Control groups of three unvaccinated mice were injected intraperitoneally with 1 LD100 of each of the 15 different serological types of *Ps. aeruginosa*.

RESULTS

Specificity of protective responses of mice after a single injection of pseudomonas vaccine

Table 2 summarizes results of experiments in which vaccinated mice were challenged on various occasions after vaccination with a range of different serological types of *Ps. aeruginosa*.

Two days after vaccination both doses of vaccine were found to have induced protection in mice against several unrelated serotypes of *Ps. aeruginosa*. The vaccine used at 1·0 mg./kg. mouse weight protected against the homologous strain and four other serotypes; there was no protection against one of these (type 9) with the lower dose of vaccine.

Table 3. *Survival of mice after challenge with* 1, 5 *and* 10 *LD* 100 *of different serotypes of* Pseudomonas aeruginosa, 4 *days after a single injection of pseudomonas vaccine P*14*F*1

Serotype of challenge strain	Size of challenge dose: 1 LD 100 Vaccine dose (mg./kg.)			5 LD 100 Vaccine dose (mg./kg.)			10 LD 100 Vaccine dose (mg./kg.)		
	1·0	0·1	0·01	1·0	0·1	0·01	1·0	0·1	0·01
1	+	+	+	−	−	−	−	−	−
2A	−	−	−	−	−	−	−	−	−
2A 2B 5C	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−
5C	−	−	−	−	−	−	−	−	−
5D	+	+	−	−	−	−	−	−	−
6A	+	+	+	+	+	+	+	+	−
6B	+	+	+	−	−	−	−	−	−
6C	+	+	+	−	−	−	−	−	−
8	+	−	−	−	−	−	−	−	−
9	+	+	+	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−
11	+	−	−	−	−	−	−	−	−
14	+	+	+	+	+	+	+	+	−
NT	−	−	−	−	−	−	−	−	−

+ = all mice survived; − = one or more mice died.

Mice challenged 4 days after vaccination showed a wider range of protection. In addition to the earlier five serotypes, they were protected against four more by the higher dose of vaccine, but against only one more by the lower dose.

At the 7th and 14th days after vaccination much of the non-specific protection was lost and mice that received either the high or the low dose of vaccine were protected against three serotypes, the homologous type 14, and types 6A and 6B.

Effect of size of challenge dose on the specificity of early protective responses

In this experiment (Table 3) three doses of vaccine, 1·0, 0·1 and 0·01 mg./kg. mouse weight, were used. Three different challenge doses of 1, 5 and 10 LD 100 were given 4 days after vaccination.

It is seen in Table 3 that the largest dose of vaccine protected mice at 4 days against 1 LD 100 of nine serotypes, the same nine that showed similar protection in Table 2 at 4 days. With 5 LD 100 there was protection against only two serotypes – the homologous type and 6A. With 10 LD 100 there was protection against the same two serotypes, but only in mice which had received the two higher vaccine doses.

Table 4. *Specificity of protective responses in mice after three injections of pseudomonas vaccine (P14F1)*

Serotype of challenge strain	Day of challenge after vaccination			
	2	4	7	14
1	+	+	+	−
2A	+	+	+	−
2A 2B 5C	−	−	−	−
3	−	−	−	−
5C	−	−	−	−
5D	+	−	−	−
6A	+	+	+	+
6B	+	+	+	+
6C	+	+	+	+
8	−	+	−	+
9	+	+	+	−
10	−	−	−	−
11	+	+	+	−
14	+	+	+	+
NT	−	−	−	−

+ = All mice survived; − = one or more mice died.

Specificity of protective responses of mice after three injections on consecutive days of pseudomonas P14F1 vaccine

The protective responses induced in mice by injection of pseudomonas P14F1 vaccine (0·1 mg./kg. mouse weight) on three consecutive days are shown in Table 4.

Two days after the third injection of vaccine mice were protected against 1 LD100 of nine serotypes. Four days after vaccination mice were still protected against nine serotypes, but had lost protection against serotype 5D and gained protection against serotype 8.

Seven days after vaccination mice were protected against eight serotypes and at 14 days after vaccination mice were still protected against five serotypes, having lost protection against only a further four serotypes of *Ps. aeruginosa*.

Specificity of protective responses in mice inoculated with different F1 vaccines

Single injections of F1 vaccines gave complete protection to mice challenged, 4 days after vaccination, with 1 LD100 of their respective homologous strains, i.e. the strains of *Ps. aeruginosa* from which the vaccines had been made (Table 5).

As well as inducing homologous protection the vaccines protected mice against 1 LD100 challenges from one or more unrelated serotypes. All vaccines induced non-specific partial protection over a wide range of serotypes by which some but not all mice were protected.

Table 5 also illustrates the potential usefulness of F1 vaccines made from different strains of *Ps. aeruginosa* in the preparation of multivalent anti-pseudo-

Table 5. *Protection of mice against* Ps. aeruginosa *infections by different pseudomonas F* 1 *vaccines*

Serotype of challenge strain	Mortality in groups of three mice vaccinated with F 1 from one of the following serotypes of *Ps. aeruginosa*							
	14	10	8	5C	3	NT	B4	2AB
14	0	1	2	1	0	0	0	2
10	1	0	2	1	1	2	3	2
8	0	1	0	2	2	3	1	3
5C	2	3	3	0	3	0	0	3
3	1	0	2	1	0	3	3	3
NT	3	2	3	1	3	0	3	3
1	0	1	2	0	2	3	1	1
2A	1	3	3	0	3	0	1	1
2A 2B 5C	3	1	0	0	3	0	2	2
5D	0	3	1	3	3	2	0	2
6A	0	1	3	0	0	1	0	0
6B	0	1	3	0	3	1	2	3
6C	0	1	1	3	3	1	2	3
9	0	3	3	3	3	3	3	0
11	0	3	3	0	3	1	0	2

monas vaccine. The F 1 vaccines made from serotypes 5C, 10, 14 and NT together induce 100 % protective responses in mice against all 15 of the challenge strains of *Ps. aeruginosa* used in these experiments.

DISCUSSION

The early protection induced in mice actively immunized with a single injection of a pseudomonas P14F1 vaccine was found not to be type-specific. Non-specific protection, following a single injection of the vaccine, appeared as early as 2 days after vaccination and was still present 14 days after vaccination. The maximum non-specific response after vaccination occurred about 4 days after vaccination when P14F1 vaccine was found to have protected mice against lethal challenges by 9/15 different serotypes of *Ps. aeruginosa*. The degree of non-specific protection induced by P14F1 vaccine was influenced by the amount of vaccine used and the frequency of vaccination; thus 4 days after vaccination a single injection of 1·0 mg./kg. mouse weight of P14F1 protected mice against challenge by three more strains of *Ps. aeruginosa* than 0·01 mg./kg. mouse weight, and a dose on three consecutive days of P14F1 vaccine protected mice against as wide a range of challenge strains, 2 and 7 days after vaccination, as a single dose of vaccine 4 days after vaccination. Preliminary studies with other F 1 vaccines made from seven unrelated serotypes of *Ps. aeruginosa* showed that protection induced 4 days after a single injection of each vaccine was also not type-specific. The non-specific protective responses induced by the vaccines 4 days after vaccination ranged from complete protection against nine serotypes of *Ps. aeruginosa* by P14F1 vaccine to complete protection against two serotypes by P8F1 vaccine. A potentially useful feature of F 1 vaccines is the speed at which protection develops after vaccination: maximum non-specific

protective responses took only 4 days to appear after a single injection of vaccine. However, the duration of this non-specific protection after a single injection of vaccine was short; for example, 3 days after the maximum non-specific protective response the mice could only resist challenge from three serotypes of *Ps. aeruginosa*, whereas 3 days earlier they had resisted challenge by nine serotypes. In another experiment it was shown that the period of maximum non-specific protection could be extended from 1 to at least 5 days by injecting the vaccine on three consecutive days. The induction of a protracted period of non-specific protection soon after vaccination shows that the F1 vaccines may provide useful anti-pseudomonas vaccines, e.g. for the protection of patients with burns, who may be simultaneously infected with different serotypes of *Ps. aeruginosa*. The experiments show that it is theoretically possible to select vaccines with wide ranges of protective activity, e.g. 5C, 10, 14 and NT, which would induce protection against all the serotypes of *Ps. aeruginosa* used in these experiments. Alexander *et al.* (1969) and Fisher, Devlin & Gnabasik (1969) have shown that combining vaccines, similar in type to F1 vaccines, has no effect on their potential immunogenic properties; combined vaccines were shown to induce protection against a wide range of *Ps. aeruginosa* strains. Previous experiments have also shown that F1 vaccines were as effective in burned mice as in unburned mice (Jones, 1971) and are of low toxicity (Jones, 1969*a*). It is possible to purify F1 vaccines by gel filtration so that only a single line of precipitation is shown by immunodiffusion (Jones, 1969*b*); purification, however, may reduce the range of types against which the vaccine will induce protection and may prove to be an unnecessary procedure.

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A comparison of the antigens present on the surface of virus released artificially from chick cells infected with vaccinia virus, and cowpox virus and its white pock mutant

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SUMMARY

Antisera prepared against vaccinia and cowpox viruses were absorbed with purified suspensions of vaccinia virus, red cowpox and white cowpox viruses. They were then tested for their ability to neutralize the viruses, and to precipitate the virus soluble antigens.

The results showed that some virus specific antigens were not virus surface components and that some components were present on the surface of all three viruses. However, certain components were detected on the surface of vaccinia virus but not on the surface of cowpox virus, and vice versa. Some evidence for the existence of a vaccinia-specific surface component was also obtained.

Comparisons between results of cross-neutralization tests and immunodiffusion tests on the absorbed sera indicated that antibody to a number of antigens, including the classical LS, and the cowpox-specific *d* antigen play no part in the process of poxvirus neutralization.

INTRODUCTION

Despite extensive studies on the serological and immunological properties of the members of the variola-vaccinia subgroup of poxviruses, their antigenic make-up is still not clear. Downie (1939*a*) and Downie & McCarthy (1950) studied these viruses using absorbed antisera. Considerable cross-reaction was found by cross-neutralization tests but the absorption of anti-vaccinia serum with cowpox virus did not reduce its specificity for vaccinia. More recently, Madeley (1968) and McNeill (1968) have detected immunological differences between vaccinia and cowpox viruses but the antigens involved have not been identified. Although any differences between these viruses must be minor when compared to the overall cross-reaction between them, such differences are of interest. Their elucidation would contribute to our knowledge of these viruses, and might in part explain their different cell tropisms and intracellular growth patterns.

The LS antigen of vaccinia is present on the surface of that virus (Craigie & Wishart, 1934, 1936; Cohen & Wilcox, 1966, 1968). However, anti-vaccinia serum absorbed with LS still agglutinates vaccinia virus, indicating that there are

additional surface antigens, the so-called 'X-agglutinogens' (Craigie & Wishart, 1938; Smadel & Shedlovsky, 1942).

Rondle & Dumbell (1962) described a cowpox-soluble antigen *d* which was absent from the white pock mutant of cowpox and possibly from vaccinia. They suggested that Downie's results (1939*a*) could be explained by postulating that *d* and LS were major surface components of red cowpox and vaccinia viruses respectively. These assumptions are tested in the present work. An attempt has also been made to determine which antigenic components of those described by Baxby & Rondle (1968) are involved in virus neutralization.

As well as comparing vaccinia and cowpox viruses the white pock mutant of cowpox was also studied, since apart from Haddock's (1952) preliminary finding that some cross-neutralization occurred between the three viruses nothing further is known about the immunological relationship of white cowpox to its parent, or to vaccinia virus.

The principal methods used have been to prepare antisera to the viruses and to absorb them with purified virus suspensions. The antisera were then tested for residual antibody by cross-neutralization tests and by immuno-diffusion.

After the antisera had been absorbed with virus artificially released from infected cells (intracellular virus), Appleyard, Hapel & Boulter (1971) and Turner & Squires (1971) reported that vaccinia virus released naturally from infected cells (extracellular virus) possessed an immunogenic component not present in virus released artificially. They also suggested that the extracellular forms of virus were more important for detecting protective antibody than the intracellular forms studied in the present paper.

MATERIALS AND METHODS

Virus strains

The Lister Institute strain of vaccinia virus, the Brighton strain of red cowpox and a white pock mutant isolated from Brighton cowpox were used primarily. Some cross-neutralization tests were done with the 61 (Gispen, 1955) and Carmarthen strains of cowpox and their white pock mutants, and the Tashkent and Connaught Laboratory strains of vaccinia virus.

Antisera

The antisera selected for absorption were a rabbit antiserum to Lister vaccinia (VS) and a rabbit antiserum to Brighton red cowpox (CS). They were prepared in conjunction with Dr C. J. M. Rondle, by initial scarification of live virus into the shaved skin of the animals, followed by repeated intravenous injections of live virus. They are known to possess antibodies to a wide range of poxvirus antigens and have been used previously to study the production of such antigens by different poxviruses (Baxby & Rondle, 1968; Baxby & Hill, 1971). Other rabbit antisera were prepared in the same way and used unabsorbed in cross-neutralization tests. Rabbit antisera were prepared against highly purified heat-inactivated virus using the immunization schedule described by Madeley (1968).

In addition, an antiserum prepared in a calf against vaccinia virus inactivated by irradiation with ultraviolet light was kindly given by Dr E. A. Boulter of the Microbiological Research Establishment, Porton.

Preparation of virus freed from soluble antigens

When preparing suspensions of purified virus care was taken to free them from contaminating soluble antigens. Briefly, the method used involved extraction of infected chorioallantoic membranes (CAM) by shaking them with glass beads, depositing the gross debris at 1000 ***g*** for 10 min., and then depositing the virus at 30,000 ***g*** for 30 min. in the SW 39 rotor of the Spinco model L centrifuge. This cycle of differential centrifugation was repeated. The resuspended virus was then extracted with Arcton 113 (I.C.I. Ltd.) and the aqueous phase banded in a sucrose-density gradient at 39,000 ***g*** for 20 min. (Zwartouw, Westwood & Appleyard, 1962). The virus, which was consistently found in the 50 % sucrose layer, was given two further cycles of differential centrifugation at 30,000 ***g*** and 1000 ***g***. At each stage the virus pellets were resuspended in 10^{-3} M-NaCl with the aid of an M.S.E./Mullard ultrasonic disintegrator.

Absorption of antisera

As antigens are known to leach from poxvirus particles into the suspending medium (Craigie & Wishart, 1936; Zwartouw, Westwood & Harris, 1965) the long absorption periods used by some previous workers were avoided. Instead, repeated short absorption periods were used as advocated by Salaman (1937). In this way it was hoped that absorption of antibody to non-surface structural components could be avoided.

Pellets of purified virus were resuspended in 1·2 ml. of antiserum and the suspensions incubated at 20° C. for 60 min. and then at 37° C. for 60 min. The virus was then deposited at 50,000 ***g***. This absorption procedure was repeated as necessary. Before testing, the absorbed antisera were spun at 50,000 ***g*** for 60 min. and the supernatant irradiated with ultraviolet light to remove and inactivate residual virus. In no instance was any residual virus detected.

Testing of absorbed antisera

The antisera were assumed to be completely absorbed when either no neutralizing antibody could be detected at a 1/30 dilution, or when additional absorption failed to reduce the neutralizing titre further. The antisera were tested for neutralizing antibody by Boulter's method (1957), in which mixtures of purified virus and serum dilutions were held at 37° C. for 2 hr. before inoculation on the CAM. When completely absorbed they were also tested for neutralizing activity against extracellular virus as described by Appleyard *et al.* (1971), using the antiserum prepared against irradiated vaccinia virus to suppress any contaminating intracellular virus.

Ouchterlony immuno-diffusion tests were done as described by Rondle & Dumbell (1962). Various preparations of soluble antigens were used, including some which contained only 1 or 2 specific line pattern components (lpc). The lpc

Table 1. *Characteristics of representative suspensions of vaccinia, red and white cowpox viruses*

Virus	Total count*	Viable count†	Total: viable	Total count/ μg. protein‡
Vaccinia	$3{\cdot}4 \times 10^9$	$2{\cdot}0 \times 10^8$	16:1	$1{\cdot}85 \times 10^8$
Red cowpox	$4{\cdot}0 \times 10^{10}$	$6{\cdot}0 \times 10^8$	66:1	$1{\cdot}7 \times 10^8$
White cowpox	$7{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^8$	70:1	$1{\cdot}75 \times 10^8$

* Counted against a reference suspension of polystyrene latex spheres (Dow Chemical Co.).
† Expressed as p.f.u./ml. on the CAM.
‡ A value of $1{\cdot}8 \times 10^8$ particles/μg. protein was obtained by Joklik (1962).

produced were identified using the reference antigens prepared and described earlier (Baxby, 1967; Baxby & Rondle, 1968). The lpc earlier referred to as 3 and 5 (Baxby & Rondle, 1968) have subsequently been shown to be the L and S antigens respectively (Rondle & Williamson, 1968) and are here so labelled.

Complement fixation tests were done as described by Macdonald & Downie (1950), except that the unit volume was 0·1 ml. Extracts of infected CAM were used as antigens either heated at 65° C. for 60 min. or unheated (Madeley, 1968).

RESULTS

The significance of the results obtained by testing absorbed antisera depends to a great extent on the purity and integrity of the virus suspensions used for absorption. Examination of purified suspensions by electron microscopy showed particles having the morphology of intact, mature poxvirus with little contaminating debris. Protein estimations gave values which were very close to those obtained by Joklik (1962) (Table 1) and the values obtained for the ratios of total/infective titres were similar to those obtained by previous workers (Table 1) (Dumbell, Downie & Valentine, 1957; Kaplan & Valentine, 1959; Joklik, 1962).

Cross-neutralization tests on antisera made against live virus

Cross-neutralization tests were performed on a series of antisera to determine whether the two selected for absorption could be regarded as 'typical'. The results (Table 2) showed that they could. High-titre neutralizing antibody was found against vaccinia and white cowpox, although titres against red cowpox were consistently lower, a feature noted earlier by Downie & McCarthy (1950).

Cross-neutralization tests on antisera made against inactivated virus

The results of cross-neutralization tests on antisera made against heat-inactivated virus are interesting as they provide information on the relative importance of heat stable antigens in virus neutralization (Table 3). The antiserum prepared against u.v.-irradiated vaccinia virus neutralized all three viruses. However, a number of antisera prepared against heated vaccinia neutralized only vaccinia virus and not cowpox viruses. This suggests that vaccinia virus has a heat-stable

Table 2. *Cross-neutralization tests on hyperimmune sera*

Serum	Virus		
	Vaccinia	Red cowpox	White cowpox
Vaccinia VS	56,000*	5,000	28,000
Vaccinia 2	50,000	6,650	44,000
Vaccinia 3	40,000	5,000	40,000
Red Cowpox CS	30,000	5,000	30,000
Red Cowpox 2	64,000	5,500	40,000
Red Cowpox 3	30,000	4,000	25,000
White Cowpox 1	15,000	3,500	17,000

* Figure is reciprocal of serum dilution giving 50 % virus survival after 2 hr. at 37° C.

Table 3. *Cross-neutralization tests on sera made against heat-inactivated virus*

Serum	Virus		
	Vaccinia	Red Cowpox	White Cowpox
Heated Vaccinia 1	18,000	25	50
Heated Vaccinia 2	3,000	20	35
Heated Red Cowpox 1	200	10,000	12,500
Heated Red Cowpox 2	75	1,000	1,250
U/V Vaccinia*	1,125,000	150,000	125,000

* Serum, supplied by Dr E. A. Boulter, prepared by inoculation of ultraviolet irradiated vaccinia virus + adjuvant into a calf.

immunogen* that is either non-immunogenic or lacking in cowpox. However, Madeley (1968) produced antisera against heated vaccinia virus which would neutralize cowpox virus. Antisera prepared against heated cowpox viruses neutralized cowpox viruses to high titre, but neutralized vaccinia virus to only very low titre. That this may be due to the different importance or amounts in these viruses of a common antigen, rather than being due to the presence of a cowpox-specific antigen, will be discussed later.

Cross-neutralization tests on absorbed antisera

Before describing the results of tests on antisera which had been completely absorbed, it is of interest to describe the unusual behaviour of sera after partial absorption with vaccinia virus (Table 4), particularly as the results obtained aid interpretation of the results obtained with antisera to heated virus. Table 4 shows that after partial absorption of VS with purified vaccinia virus, the serum had a much lower titre against cowpox viruses than against the absorbing virus. As discussed later, this suggests that shared antigens may be more important for neutralization of cowpox virus than vaccinia virus.

Tables 5 and 6 show cross-neutralization tests on CS and VS respectively after complete absorption; a concentration of 1/30 was the highest that could be used

* Immunogen = an antigen which stimulates the production of virus-neutralizing antibody.

Table 4. *Cross-neutralization tests on vaccinia antiserum VS after partial absorption with vaccinia virus*

	Serum VS absorbed with	
Test virus	Nil	Vaccinia
Vaccinia	56,000	30,000
Red Cowpox	5,000	100
White Cowpox	28,000	250

Table 5. *Cross-neutralization tests on cowpox antiserum CS after complete absorption with purified virus*

	Absorbing virus			
Test virus	Nil	Vaccinia	Red cowpox	White cowpox
Vaccinia	30,000	< 30	< 30	< 30
Red cowpox	5,000	< 30	< 30	< 30
White cowpox	30,000	< 30	< 30	< 30

Table 6. *Cross-neutralization tests on vaccinia antiserum VS after complete absorption with purified virus*

	Absorbing virus			
Test virus	Nil	Vaccinia	Red cowpox	White cowpox
Vaccinia	56,000	< 30	25,000	21,000
Red cowpox	5,000	< 30	< 30	< 30
White cowpox	28,000	< 30	< 30	< 30

with the small volumes of antisera available. The results with vaccinia and red cowpox confirm and extend those obtained by Downie & McCarthy (1950), who did only qualitative tests for residual antibody, and provide data for white cowpox virus which has been described since then.

Absorption of the anti-cowpox serum with each of the three viruses removed all detectable neutralizing antibody to them. This result suggests that the neutralizing antibodies induced by infection with red cowpox correspond to antigens present on the surface of all three viruses.

Absorption of the anti-vaccinia serum VS with vaccinia virus removed all detectable neutralizing antibody for all three viruses, but absorption of VS with either red or white cowpox left high titres of vaccinia-neutralizing antibody. This result suggests that vaccinia virus elicits a neutralizing antibody to a vaccinia virus surface immunogen which is absent from the surface of both red and white cowpox viruses. Comparison of this result with the results obtained with antisera to heat-inactivated and irradiated vaccinia virus suggests that this vaccinia specific immunogen is heat-stable.

These results on absorbed antisera provided no evidence of any qualitative differences between the surface immunogens of red and white cowpox viruses.

Table 7. *Cross-neutralization tests on antiserum VS after complete absorption with intracellular virus, using extracellular test virus**

Test virus (extracellular)	Antiserum absorbed with intracellular			
	Nil	Vaccinia	Red cowpox	White cowpox
Vaccinia	40,000	28,000	32,000	30,500
Red cowpox	4,300	3,200	2,700	2,950
White cowpox	27,000	19,000	22,000	18,000

* Neutralization tests were done in the presence of 1/500 antiserum to irradiated vaccinia virus, which suppressed any contaminating intracellular virus.

Cross-neutralization tests with extracellular virus on antisera absorbed with intracellular virus

After the appearance of the papers by Appleyard *et al.* (1971) and Turner & Squires (1971) on the properties of extracellular vaccinia virus, all the absorbed antisera were tested for neutralizing antibody to suspensions of extracellular vaccinia, red and white cowpox viruses. In all instances high-titre neutralizing antibody to extracellular virus was found. The results obtained with VS are shown in Table 7; similar results were obtained with the anti-cowpox serum. This not only confirms the above workers' finding that vaccinia antisera absorbed with intracellular vaccinia virus still contained neutralizing antibody for extracellular virus, but also indicates that this also occurs with cowpox viruses.

Experiments with other strains of vaccinia and cowpox virus

There are many strains of vaccinia virus which can be distinguished by suitable laboratory tests (Fenner, 1958). There is also accumulating evidence that strains of cowpox virus may be differentiated. Kato, Takahashi, Kameyama & Kamahora (1959) showed that the 'A' inclusion of cowpox, originally described for the Brighton strain by Downie (1939*b*), in some strains contains no virus particles (V^-, e.g. Brighton) and in other strains has virus particles embedded in it (V^+). We have recently described a third alternative (V^i) where the periphery of the inclusion is covered with virus particles, but no particles are present within the inclusion (Plate 1A) (D. Baxby & D. R. Moorcroft, unpublished). The possibility that immunological differences might exist among different strains of either vaccinia or cowpox was tested by doing neutralization tests on absorbed antisera with the Tashkent and Connaught Laboratories strains of vaccinia, and the Carmarthen (V^+) and 61 (V^i) strains of cowpox virus. The results obtained showed no evidence of immunological strain differences.

Complement fixation tests on absorbed antisera

Complement fixation tests were done on all absorbed antisera using heated and unheated antigens. In no case, however, were complement-fixing antibodies completely absorbed. This result becomes understandable when one considers the complexity of poxvirus soluble antigens and the fact that it is uncertain how many of these antigens fix complement. If the antibody with the highest complement-

Table 8. *Surface antigens of intracellular vaccinia and cowpox viruses detected by immuno-diffusion tests on absorbed sera*

	Antigens										
Virus	1	2	3 (L)	4	5 (S)	6	7	8	9	10	*d*
Vaccinia	−	−	+	+	+	−	+	−	−	+	−
Red cowpox	−	−	−	+	−	−	−	+	+	+	+
White cowpox	−	−	−	+	−	−	−	+	+	+	−

\+ = Antigen present on virus surface, i.e. absent from absorbed antiserum.
− = Antigen absent from virus surface.

fixing titre is specific for a non-surface antigen, exhaustive absorption with purified virus should not reduce the titre of the antiserum. If the antibody with the highest complement-fixing titre is specific for a surface antigen, absorption will reduce the overall complement-fixing titre to that determined by the highest concentration of antibody to non-surface antigens. The results obtained support the view that only antibody to surface antigens had been absorbed.

Immuno-diffusion tests on absorbed antisera

Ouchterlony immuno-diffusion analysis of the absorbed antisera was carried out in order to identify the line pattern components to which residual antibody was present. Although unequivocal results were only obtained for 11 of the components, these results were clear-cut and are summarized in Table 8. Representative experiments are also illustrated (Plate 1 B–G).

Absorption with vaccinia virus removed antibody to LS, indicating that these antigens are present on the surface of that virus (Plate 1 B). However, absorption with either red or white cowpox did not remove antibody to these components, indicating the absence of LS from the surface of these viruses (Plate 1 C). Absorption of CS with red cowpox virus removed antibody to lpc *d*, indicating the presence of this antigen on the surface of red cowpox (Plate 1 D). However, absorption with either vaccinia or white cowpox did not remove antibody to lpc *d* (Plate 1 E), indicating that this component is absent from the surface of vaccinia and white cowpox viruses. Thus Rondle & Dumbell's (1962) hypothesis regarding the location of these components is confirmed; their significance in virus neutralization will be discussed later.

Further tests indicated that lpc 1, 2 and 6 were not virus surface components whereas lpc 4 and 10 were (Plate 1 D–G). An interesting situation was found with respect to lpc 8, 9. These components were detected on the surface of red and white cowpox viruses, but not on the surface of vaccinia virus (Plate 1 F, G). This result is the reverse of that obtained with LS.

DISCUSSION

These results relate to the antigens present on the surface of vaccinia and cowpox viruses extracted from infected cells. The immunological response to poxvirus infection and the role of antibody in recovery from infection have not been

considered. The immunological response to poxvirus infection has been studied extensively since Downie (1939*a*) showed that cowpox and vaccinia viruses were not identical, and the recent work of Boulter (Boulter, 1969; Appleyard *et al.* 1971) and Turner & Squires (1971) suggests that antibody to extracellular virus may be more important than antibody to intracellular virus in recovery from infection. Nevertheless intracellular forms of poxvirus are neutralized by antisera, and it is from the study of intracellular virus that most of our knowledge of the serology and chemistry of poxviruses has been obtained. Owing to the difficulties involved in obtaining high concentrations of extracellular virus free from intracellular virus (Appleyard *et al.* 1971), intracellular virus will probably continue to be used for some purposes.

The immuno-diffusion analysis of the antisera after absorption with purified virus indicates clearly that although vaccinia and red and white cowpox viruses share some surface components the surfaces of the three viruses are not identical. Thus the LS antigens and lpc 7, with which they are often associated (Baxby, 1967), are present on the surface of vaccinia virus but not of the two cowpox viruses. In contrast, lpc 8 and 9 are present on the surface of red and white cowpox virus but not vaccinia. Antibody to lpc LS, 7, 8 and 9 are present in antisera prepared against all the viruses and lpc 8 and 9 are present as soluble antigens of all the viruses (Baxby & Rondle, 1968). However, the location of LS and 7 in cowpox has not been established, although Rondle & Dumbell (1962) extracted what is now known to be LS (D. Baxby & E. C. Smith, unpublished) from cowpox-infected tissues with trypsin. Lpc 1, 2 and 6 are not surface antigens of these viruses whilst lpc 4 and 10 are. Thus lpc 4 and 10 can be counted among the 'X-agglutinogens'.

When this distribution of surface antigens is compared with the results of neutralization tests some conclusions can be drawn about the role of different antibodies in virus neutralization. Obviously antibody to antigens such as lpc 1, 2 and 6 which are not surface components of intracellular virus can play no part in the neutralization of that virus. However, one or more of these antigens may represent the additional immunogenic component of extracellular virus described recently (Appleyard *et al.* 1971; Turner & Squires, 1971). Extracellular forms of vaccinia and cowpox viruses were neutralized by absorbed antisera, indicating that the extracellular forms of all three viruses share an additional common antigen, or antigens.

In general, the results obtained indicated that antibody to lpc L, S, *d*, 8 and 9 plays no role in the neutralization of intracellular vaccinia or cowpox viruses. Antiserum containing antibody to LS, which has been absorbed with cowpox virus, still contains anti-LS but will not neutralize vaccinia. White cowpox antisera do not have anti-*d* but will neutralize red cowpox. Also a serum containing anti-*d* which has been absorbed with white cowpox still contains anti-*d* yet will not neutralize red cowpox. An antiserum containing antibody to lpc 8, 9 which has been absorbed with vaccinia still contains anti-8, 9 but will not neutralize cowpox.

Attempts to obtain vaccinia-neutralizing antibody by immunization with LS preparations have produced varied results. Parker & Rivers (1936) and Woodroofe & Fenner (1962) were unsuccessful while Cohen & Wilcox (1966) although initially

unsuccessful later succeeded (Wilcox & Cohen, 1968). Although the LS complex can be shown by immuno-diffusion analysis to exist in its classical form, i.e. as two antigenic sites on one molecule (Rondle & Williamson, 1968), on purification it is invariably contaminated with other virus antigens (Marquardt, Holme & Lycke, 1965; Cohen & Wilcox, 1966), and it is possibly antibody to these extra antigens which neutralizes the virus.

Lpc 4 and 10 were surface components common to all three viruses and so could be neutralization sites. However, Appleyard & Westwood (1964) and Westwood, Zwartouw, Appleyard & Titmuss (1965) have detected up to 20 poxvirus antigens compared to the 11 for which results are presented here, and it is possible that there are additional common surface immunogens.

Experiments on the neutralization of poxviruses by antisera prepared against heat-inactivated virus provide information that common heat-stable antigens may vary in importance in the neutralization of different viruses, and also provide evidence for a heat-stable vaccinia specific antigen.

Antisera prepared against heated cowpox virus neutralized cowpox virus to high titre, but vaccinia virus to very low titre. However, as immune cowpox serum absorbed with vaccinia virus will not neutralize cowpox virus, the heat-stable antigen involved is not cowpox specific. This suggests that this common heat-stable antigen is relatively unimportant for the neutralization of vaccinia virus. An anti-vaccinia serum partially absorbed with vaccinia virus had very low-titre neutralizing antibody for cowpox virus, but high-titre neutralizing antibody for vaccinia virus. This also suggests that vaccinia has a major surface antigen which is more important for the neutralization of cowpox virus than vaccinia virus.

Antisera prepared against heat-inactivated vaccinia virus neutralized vaccinia virus but not cowpox virus. This suggests that the common heat-stable component discussed above is haptenic or non-immunogenic in vaccinia virus. Neter (1969) has described how a common specificity may be immunogenic in one organism and haptenic in a closely related organism. It also suggests that vaccinia virus is neutralized through a heat-stable antigen which is either absent or non-immunogenic in cowpox virus. After absorption with cowpox virus a vaccinia antiserum still neutralizes vaccinia, as originally shown by Downie (1939*a*) and Downie & McCarthy (1950), a result which suggests that the heat-stable immunogen may be absent from the surface of cowpox virus. However, Madeley (1968) produced antisera against heated vaccinia which did neutralize cowpox virus; the reason for this discrepancy is not known. The virus in Madeley's vaccine was not so rigorously purified and contained large amounts of, for example, poxvirus haemagglutinin (HA). This extra material may have protected one of the common heat-labile immunogens, and antibody to such a component may have neutralized cowpox virus. Certainly the heat resistance of some poxvirus antigens depends on their degree of purity. The purification of HA produces material which becomes progressively less heat resistant and less antigenic (Madeley, 1968; E. C. Smith & D. Baxby, unpublished). It is possible that a similar situation exists with immunogenic components.

The results obtained did not suggest that there are qualitative immunological

differences between red and white cowpox viruses, although the absence in white cowpox of the non-immunogenic component *d* has been noted. The lower titres found against red cowpox may indicate differences in the amounts or importance of shared antigens.

Examination of other strains of vaccinia and cowpox provided no evidence of serological strain differences. This result is in agreement with the work of Downie (1939*a*), Horgan & Haseeb (1945), Gispen (1955) and Rondle & Dumbell (1962). However, such is the variety of biological properties exhibited by different strains of vaccinia virus (Fenner, 1958) that the existence of serologically distinct strains cannot be ruled out.

The results presented here emphasize, as have those of other workers, that the neutralization of even intracellular pox viruses is not a simple process. The presence of certain antigens on the surface of these viruses has been determined and although it has not proved possible to identify the immunogenic components of these viruses some surface antigens, including the LS antigen, have been shown to be non-immunogenic. Despite the large degree of cross-reaction between vaccinia and red and white cowpox they are not immunologically identical. They appear to be neutralized through common heat-labile antigens, and there is evidence that heat-stable common antigens may be more important for the neutralization of cowpox than vaccinia, and that vaccinia may have a specific heat-stable immunogen. The additional immunogens acquired by extracellular virus still require identification.

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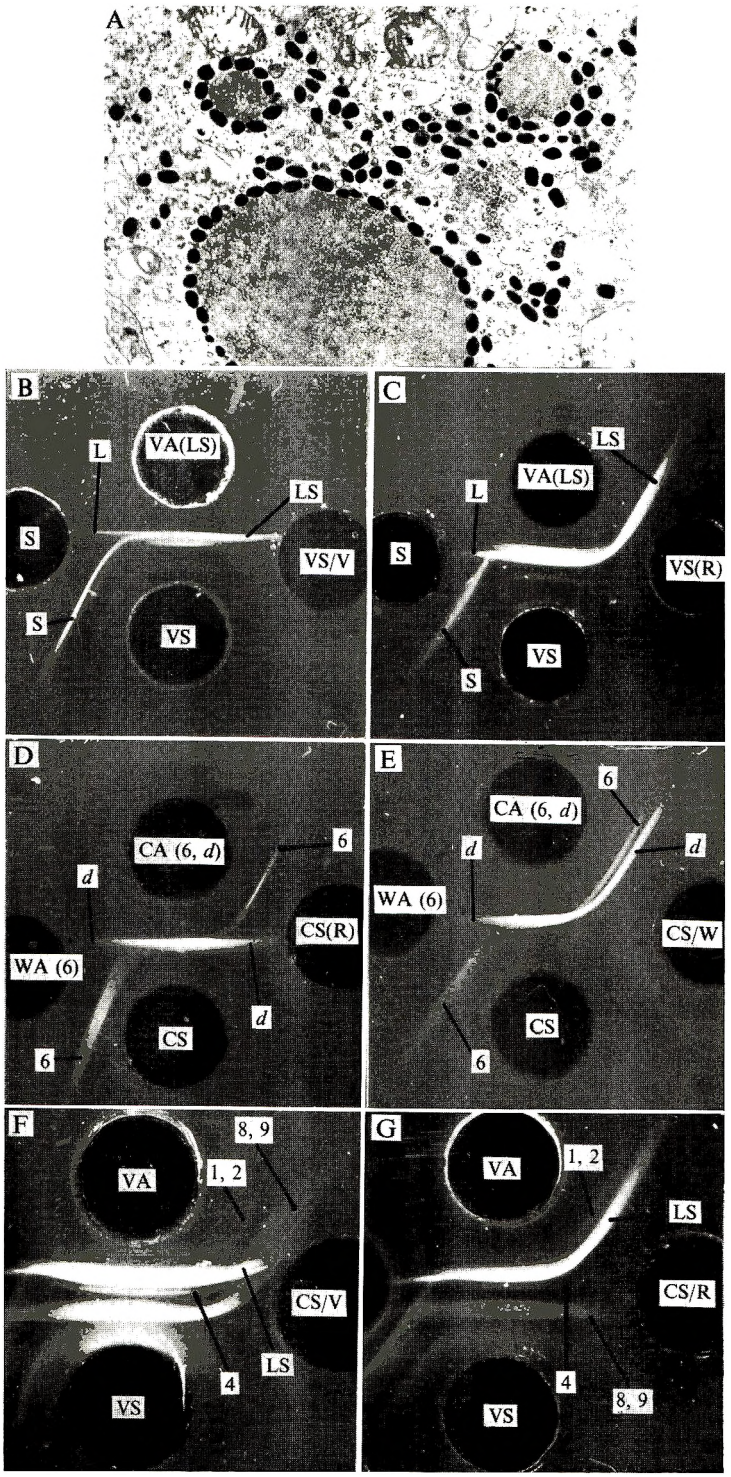
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8, 9
VS

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EXPLANATION OF PLATE

Fig. A. Electron micrograph of section of 'A' inclusion produced by cowpox strain 61, showing the virus particles embedded round the periphery of the inclusion. × 11,000.

Fig. B. Absorption of antivaccinia serum (VS) with vaccinia virus (VS/V) removes antibody to LS. VA(LS) = vaccinia soluble antigen containing essentially only LS. S = heated LS containing essentially only S.

Fig. C. Absorption of VS with red cowpox (VS/R) does not remove antibody to LS.

Fig. D. Absorption of anticowpox serum (CS) with red cowpox virus (CS/R) removes antibody to *d* but not to 6. CA (6, *d*) = Red cowpox soluble antigen specific for 6 and *d*. WA (6) = White cowpox antigen specific for 6.

Fig. E. Absorption of CS with white cowpox virus (CS/W) does not remove antibody to 6 or *d*.

Fig. F. Absorption of CS with vaccinia virus (CS/V) removes antibody to LS, 4, but not to 1, 2, 8, 9. VA = complex vaccinia soluble antigen. VS = vaccinia antiserum.

Fig. G. Absorption of CS with red cowpox (CS/R) removes antibody to 4, 8, 9, but not 1, 2, LS.

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An evaluation of strontium chloride, Rappaport and strontium selenite enrichment for the isolation of salmonellas from man, animals, meat products and abattoir effluents

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SUMMARY

Strontium chloride enrichment broth was found to be comparable to Rappaport broth for the recovery of a wide range of *Salmonella* serotypes from man, animals, meat products and effluents. With the exception of cloacal samples from reptiles, both procedures were superior to selenite F.

The performance of strontium chloride M and selenite F enrichment was improved when effluent samples were incubated at 43° C.

Strontium chloride M and Rappaport enrichment were superior to selenite F for the isolation of *Arizona* species from reptiles.

Strontium chloride B, strontium selenite and Rappaport broths were found suitable for the isolation of multiple *Salmonella* serotypes from sea water contaminated with abattoir effluents. The strontium chloride B and strontium selenite enrichment media were superior to Rappaport broth when samples were incubated at 43° C.

Modified bismuth sulphite agar was found superior to Salmonella–Shigella agar as a solid subculture medium.

The investigation of a food poisoning outbreak due to *Salmonella typhimurium* phage type 21 is reported.

The significance of the choice of sampling and isolation techniques in salmonellosis in man and animals is discussed.

INTRODUCTION

Meat products have often been implicated as a vehicle of salmonella infection, furthermore it has been demonstrated that there is a close relationship between the types of *Salmonella* isolated from abattoirs and meat processing centres, and from human cases in the same area (Galton, Smith, McElrath & Hardy, 1954; McDonagh & Smith, 1958; Harvey & Phillips, 1961). Assessments of the frequency of salmonella infection have shown wide variation, and Schothorst & Kampelmacher (1967) and Vassiliadis, Trichopoulos, Papadakis & Politi (1970) have attributed differences to slaughterhouse practice, meat processing procedures, and methods of laboratory examination.

The selenite F enrichment medium of Leifson (1936) and the tetrathionate broth of Muller (1923) are widely used for the isolation of salmonellas. Important modifications include the tetrathionate broth of Kauffmann (1930, 1935) and the selenite broth of Hobbs & Allison (1945).

The inhibitory action of chlorides on the growth of certain Enterobacteriaceae was first investigated by Eisenburg (1918) and Hotchkiss (1923). Subsequently, lithium chloride was used by Gray (1931), magnesium chloride by Rappaport, Konforti & Navon (1956), and strontium chloride by Iveson & Mackay-Scollay (1969) and Iveson (1971), as selective ingredients in salmonella isolation media.

In trials of Rappaport broth, Collard & Unwin (1958), Iveson, Kovacs & Laurie (1964), Hooper & Jenkins (1965) and Iveson & Kovacs (1967) demonstrated improved salmonella recoveries over both selenite and tetrathionate media. The medium was also found suitable for the isolation of the selenite and tetrathionate sensitive *S. cholerasuis* from pigs by Iveson & Mackay-Scollay (1969), Riley (1970) and Beh (1971). On the other hand, Harvey & Price (1968) found Rappaport broth less suitable for the isolation of *S. dublin* and *S. pullorum*. The medium was also not suited to incubation at 43° C., a temperature effective in improving the performance of selenite F broth (Harvey & Thomson, 1953).

In comparative trials of two new enrichment media containing strontium ion, Iveson & Mackay-Scollay (1969) observed that in tests with sewage and human and swine faeces, strontium chloride M medium compared favourably with Rappaport broth in the isolation of salmonellas and, except for the isolation of *S. typhi*, was superior to selenite F. They also found that strontium selenite broth was superior to Rappaport, strontium chloride M, tetrathionate and selenite F for the isolation of *S. typhi*.

Similar results were obtained by Chau & Huang (1971), who recommended the combination of strontium selenite and Rappaport broths for the isolation of *Salmonella*. However, the methods quoted were unsuitable for the isolation of *Edwardsiella tarda*, and Iveson (1971) introduced strontium chloride B broth for the improved isolation of *Edwardsiella* as well as *Salmonella* and *Arizona* species. The new medium was also well suited to the elevated temperature technique.

In the present study an evaluation of the relative performance of strontium chloride M, strontium chloride B, strontium selenite, Rappaport and selenite F enrichment media was undertaken during a survey of salmonella infection in animals, reptiles and poultry, in a study of abattoir effluents and in an investigation of a food-poisoning outbreak. The methods of examination and results obtained are presented in this report.

MATERIALS AND METHODS

The investigation comprised three complementary studies. The first, Study I, involved the testing of 940 specimens from cattle, pigs, sheep, poultry, rats, mice, guinea-pigs, dogs, cats, snakes, lizards and ticks. Strontium chloride M, Rappaport and selenite F enrichment media were used to examine samples of faeces, glands, intestinal contents and viscera. Study II consisted of the investigation of a

salmonella food-poisoning outbreak in humans. The same enrichment media were used to examine a total of 1541 samples which comprised human faeces, raw and cooked meats and the effluents from meat-processing centres and abattoirs. Study III was directed particularly to the isolation of multiple *Salmonella* serotypes from 18 samples of sea water heavily contaminated with abattoir effluent using strontium chloride B, strontium selenite and Rappaport enrichment broth media, incubated at 37 and 43° C.

Study I

With the exception of cloacal swabs from reptiles, which were placed direct into 2 ml. of Sachs (1939) transport medium, all samples were homogenized in equal volumes of the transport solution and inoculated on SS agar (Oxoid) and modified bismuth sulphite agar (Iveson, 1971). Approximately 0·5 ml. volumes of each sample were added to 10 ml. volumes of strontium chloride M, Rappaport and selenite F enrichment media respectively and incubated at 37° C. Subcultures were made from enrichment media to one SS agar and one modified BS agar plate after 18–24 hr incubation.

Study II

Faeces and effluent samples were collected as described by Iveson & Mackay-Scollay (1969). The surface swabs of meat and meat-processing equipment were collected on folded gauze swabs held with sterile forceps and immediately placed into 100 ml. of $\frac{1}{4}$ strength Ringer's solution. After mixing, 15 ml. volumes were added to 150 ml. volumes of the three enrichment media, strontium chloride M, Rappaport and selenite F broths which were incubated at 37° C. In addition, selected meat processing and abattoir effluents were also inoculated into strontium chloride M and selenite F enrichment media and incubated at 43° C. Meat and effluent samples were subcultured to SS and modified BS agar at 24 and 48 hr incubation. Faeces samples were similarly subcultured but only after 24 hr incubation.

Study III

Samples of sea water heavily contaminated with effluent were collected at nine sampling points close to the shoreline, and not farther than half a mile from the abattoir effluent outflow. Moore swabs were immersed for approximately 6 hr., lifted and replaced by swabs left overnight at the sampling points.

Samples were transported to the laboratory in 100 ml. $\frac{1}{4}$ strength Ringer's solution, the contents mixed and 15 ml. inoculated into 150 ml. quantities of strontium chloride B, Rappaport and strontium selenite enrichment media, and incubated at 37 and 43° C. The remaining fluid was drained from the container and the swab (which contained approximately 15 ml. retained fluid) was covered with 150 ml. strontium chloride B broth and incubated at 43° C. Each sample was subcultured to a plate of SS and one of modified BS agar after 24 hr. incubation, and to a BS agar plate only at 48 hr. In addition, 14 of the samples were subcultured to BS agar after 72 hr. incubation.

Media

Strontium chloride M enrichment broth

Bacto tryptone (Difco)	0·5 g.
Sodium chloride	0·8 g.
Potassium dihydrogen phosphate	0·1 g.
60 % strontium chloride (B.D.H.)	6·0 ml.
0·4 % malachite green (Merck)	1·0 ml.
Distilled water	100 ml.

The medium was distributed in 10 ml. or 150 ml. volumes as required, and sterilized by steaming for 30 min. The final concentration of strontium chloride was 3·4 % and the pH was 5·0–5·5.

Strontium selenite enrichment broth

Bacto tryptone (Difco)	0·5 g.
Sodium chloride	0·8 g.
Strontium hydrogen selenite (Ajax)	0·2 g.
Potassium dihydrogen phosphate	0·1 g.
Distilled water	100 ml.

The broth was adjusted to pH 6·8, distributed in 150 ml. amounts and steamed for 30 min. The strontium hydrogen selenite salt, supplied by Ajax Chemicals Ltd. of Australia, replaced the salt previously prepared in our laboratories (Iveson & Mackay-Scollay, 1969).

Other enrichment broth media

The strontium chloride B, Rappaport and selenite F media were prepared as reported by Iveson (1971).

An average of three suspect colonies were selected from each of the plating media and examined biochemically and serologically.

Supplementary sero-recovery technique.

In addition to routine colony identification, the BS agar plates from the 24 hr. subculture routine in Study III were examined for the presence of further *Salmonella* serotypes by a modification of the serological recovery method of Harvey & Price (1967). Swabs of positive BS plates were inoculated to Craigie tubes which contained the homologous flagellar phase 1 and phase 2 antisera of the serotypes previously identified from the same plates. After overnight incubation, growth from Craigie tubes was subcultured to BS agar and colonies examined in the usual way.

Table 1. *The relative efficiency of direct culture and selenite F, Rappaport and strontium chloride M enrichment in the isolation of* Salmonella *from domestic animals, rodents and reptiles*

Samples		Tests	Culture method							
			D	S	R	C	S+R	S+C	R+C	S+C+R
Livestock	tissues	348	20*	38	50	50	51	51	56	57
	faeces	371	17	52	68	78	82	90	90	97
	rumen	10	2	4	4	7	5	7	7	7
Domestic pets faeces		32	0	3	11	13	12	14	14	14
Rodents faeces		83	5	11	28	27	28	28	31	31
Reptiles faeces		82	28	47	58	59	64	62	67	68
Reptiles ticks		14	0	3	2	4	4	4	5	5
Totals		940	72	158	221	238	246	256	270	279
Efficiency (%)			25·8	56·6	79·2	85·3	88·2	91·8	96·8	100

* The figures show the number of positive cultures.
D = direct culture; S = selenite F; R = Rappaport; C = strontium chloride M.

RESULTS

A total of 864 isolations which comprised 828 *Salmonella*, 35 *Arizona* and 1 *Edwardsiella* strain were isolated from 631 positive samples. *Salmonella typhimurium* was most frequently isolated and was recovered from 248 (39 %) of the positive samples. A total of 51 *Salmonella* and 14 *Arizona* serotypes were identified. The distribution of the *Salmonella* serotypes is shown in Table 5.

In tests on animals (Study I) a total of 340 strains comprising 306 *Salmonella*, 33 *Arizona* and 1 *Edwardsiella* strain, were isolated from 279 positive specimens. Of the positive samples 72 (26 %) were obtained by direct culture and 279 (100 %) by enrichment culture. The enrichment media recovery totals were strontium chloride M 238 (85 %), Rappaport 221 (79 %) and selenite F 158 (57 %). The relative efficiency of direct culture and enrichment methods is shown in Table 1. In tests on cattle, sheep and pig samples there was no significant difference in the performance of strontium chloride M and Rappaport broths, and both procedures were superior to selenite F.

Similar results were obtained in tests on rodent, dog, cat and snake samples. Two possible exceptions were noted in the lizard and poultry test series, where selenite F was comparable to Rappaport medium in performance and only slightly inferior to strontium chloride M broth.

In the course of the animal investigations, 45 *Salmonella*, 13 *Arizona* and *Edwardsiella tarda* serotype recoveries were made.

Figures in Table 2 show that in the recovery of Arizonas, strontium chloride M and Rappaport enrichment methods were each superior to either direct culture or selenite F methods. In fact, from the 18 reptiles where *Arizona* species were recovered exclusively only six were positive by a combination of direct culture and selenite enrichment, compared with 15 recoveries through Rappaport and 13 through strontium chloride M enrichment broths. Conversely, in the 35 reptiles

Table 2. *Relative efficiency of strontium chloride M, Rappaport and selenite F enrichment cultures and direct culture in the isolation of* Salmonella, Arizona *and* Edwardsiella *species from 54 reptiles*

	Salmonella		*Arizona*		*Edwardsiella*	
Culture method	No. positive	Efficiency (%)	No. positive	Efficiency (%)	No. positive	Efficiency (%)
Strontium chloride M	32	91·4	13	72·2	0	.
Rappaport	29	82·9	15	83·3	0	.
Selenite F	32	91·4	6	33·3	0	.
Direct culture	17	48·6	6	33·3	1	.
Totals	35	100	18	100	1	.

Table 3. *The relative efficiency of direct culture and selenite F, Rappaport and strontium chloride M enrichment in the isolation of* Salmonella *from human faeces, meats, meat factories and abattoirs*

		Culture method							
Samples	Tests	D	S	R	C	S+R	S+C	R+C	S+C+R
Human faeces	1111	88	142	155	161	164	167	168	172
Meats	177	.	27	34	29	39	41	39	42
Effluents { Meat factory	63	.	9	12	14	20	17	22	24
Effluents { Abattoir	52	.	18	45	45	45	46	52	52
Swabs { Meat	62	.	7	14	13	16	15	18	19
Swabs { Equipment	58	.	5	10	13	15	15	19	19
Totals	1523	.	208	270	275	299	301	318	328
Efficiency, %	.	.	63·4	82·3	83·8	91·2	91·8	97·0	100

Notes as in Table 1.

which yielded *Salmonella* exclusively, the performance of the three enrichment methods were comparable though direct culture was again greatly inferior. However, the single *Edwardsiella* isolation was made exclusively from direct culture.

The following *Arizona* serotypes were recovered from reptiles; the figures in parentheses indicate the frequency of isolations: *Arizona* 5:29–21 (1); 5:29–30 (1); 15:24–31 (1); 16:23–25 (9); 16:23–37 (3); 16:27–25 (1); 26:24–25 (1); 26:33–31 (4); 28:23–25 (1); 28:32–28 (2); 29:29–25 (2); 30:26–21 (2); 1, 33:23–21 (4). *Arizona* 1, 33:23–21 was also recovered from ticks taken from one reptile.

In the food-poisoning investigations, Study II, a total of 419 isolations comprising 417 *Salmonella* and 2 *Arizona* strains were isolated from 328 positive samples. The enrichment media recovery totals were, strontium chloride M, 275 (84%); Rappaport, 270 (82%); and selenite F, 208 (63%) positive samples. The relative performance of the isolation methods used is shown in Table 3.

In tests on human faeces, strontium chloride M and Rappaport enrichment were slightly superior to selenite F enrichment when the broths were incubated at 37° C. Similar results were obtained in tests on meat products, meat swabs and meat

Table 4. *Relative efficiency of selenite F and strontium chloride M enrichment at* 37° *and* 43° *C. and Rappaport enrichment at* 37° *C. in the isolation of* Salmonella *from* 30 *meat factory and* 39 *abattoir effluent samples*

	Culture method and isolation										
	37° C.							43 °C			
Effluent	S	C	R	SC	SR	RC	SCR	S	C	SC	All methods
Meat factory	9	14	14	18	20	22	24	12	19	21	30
Abbattoir	15	34	35	35	35	39	39	28	39	39	39
Totals	24	48	49	53	55	61	63	40	58	60	69
Efficiency (%)	34·8	69·9	71·0	76·0	79·7	88·4	91·3	58·0	84·1	87·0	100·0

Table 5. Salmonella *serotypes isolated from all sources in Studies I, II and III*

Salmonella serotype	Source and isolations: Human faeces	Live-stock	Abat-toir	Meat pro-cessing	Kangaroo meat	Domestic pets	Rodents	Reptiles	Totals
abony	.	3	.	.	.	.	.	.	3
adelaide	3	31	24	3	3	7	.	1	72
alsterdorf	.	.	.	.	.	.	.	1	1
anatum	.	2	8	5	5	3	.	.	23
bahrenfeld	.	2	3	.	.	.	.	.	5
birkenhead	.	1	.	.	.	.	.	.	1
boeker	.	.	.	.	.	.	.	1	1
bootle	.	.	.	.	.	.	.	1	1
bovismorbificans	.	5	10	2	.	.	.	.	17
bredeney	.	4	12	.	.	.	.	.	16
bukavu	.	.	1	.	.	.	.	.	1
bullbay	.	.	.	.	.	.	.	4	4
champaign	.	1	.	.	.	.	.	1	2
charity	.	.	.	.	1	.	.	1	2
chester	1	48	11	1	2	1	1	5	70
cholerae-suis	.	11	.	.	.	.	.	.	11
derby	.	18	61	1	.	.	.	.	80
eastbourne	.	.	1	.	.	.	.	.	1
eimsbuettel	.	.	2	.	.	.	.	.	2
emmastad	.	.	.	.	.	.	.	1	1
enteritidis	.	.	.	.	.	.	5	1	6
give	.	4	10	1	.	1	.	4	20
havana	4	.	18	.	.	.	.	.	22
houten	.	.	.	.	2	.	.	. (1)	3
hvttingfoss	.	.	1	.	.	2	.	1	4
litchfield	.	2	.	.	.	.	.	.	2
livingstone	.	.	14	.	.	.	.	.	14
kibusi	.	.	.	.	.	.	.	2	2
kisarawe	.	.	.	.	.	.	.	3	3

Table 5 (*cont.*)

Salmonella serotype	Source and isolations: Human faeces	Live-stock	Abat-toir	Meat pro-cessing	Kangaroo meat	Domestic pets	Rodents	Reptiles	Totals
muenchen	5	5	4	4	19	.	22	2	61
nashua	.	.	.	.	.	.	.	14	14
new-brunswick	.	1	.	.	.	.	.	.	1
newington	.	1	6	7	1	1	.	1	17
onderstepoort	.	4	.	.	2	.	.	.	6
oranienburg	.	3	5	.	.	.	.	.	8
orientalis	.	.	.	.	.	.	.	2 (1)	3
orion	1	6	2	2	5	.	.	.	16
ohlstedt	.	.	.	.	.	.	.	2 (1)	3
potsdam	.	.	1	.	1	1	.	.	3
pullorum	.	6	.	.	.	.	.	.	6
rubislaw	3	.	.	.	.	.	.	.	3
saint-paul	.	7	.	.	.	2	2	5	16
senftenberg	.	.	7	2	.	.	1	.	10
sydney	.	.	.	.	.	.	.	. (1)	1
tennessee	.	1	4	3	.	.	.	.	8
typhimurium	154	19	18	47	2	.	7	1	248
urbana	.	2	.	.	.	.	.	.	2
wandsbek	.	.	3	.	.	.	.	4	7
waycross	.	.	.	.	2	.	.	.	2
welikada	.	.	.	.	2	.	.	.	2
42 : z : –	.	.	.	.	.	.	.	1	1
Totals	171	187	226	78	47	18	38	59 (4)	828

Figures in parentheses indicate isolations from ticks on reptiles.

Table 6. *Distribution and relative frequency of* Salmonella *serotypes isolated by five procedures from* 18 *samples of sea water contaminated with abattoir effluent*

Method	Temperature °C	Incubation (hr.)	Plating medium	Colony tests No.	Colony tests Pos.	*livingstone*	*derby*	*bredeney*	*typhimurium*	*give*	*havana*	*senftenberg*	*adelaide*	*bovismorbificans*	*bahrenfeld*	*wandsbek*	*anatum*	*bukanu*	*chester*	*kwitingfoss*	*muenchen*	*newington*	Isolations			Positive samples		
I. Strontium selenite	37	24	SS	40	3	+	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	3	20	41	2	13	16
			BS	47	23	+	+	+	.	+	+	.	+	.	.	.	.	.	.	.	.	.	15			11		
		48	BS	21	10	.	+	.	+	+	+	.	+	.	.	+	.	.	.	.	.	.	8			7		
	43	24	SS	40	22	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	.	.	14	35		13	16	
			BS	42	35	+	+	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	23			16		
		48	BS	30	25	+	+	+	+	+	+	.	+	.	+	.	.	.	+	.	.	.	20			13		
II. Rappaport	37	24	SS	27	19	+	.	+	+	+	.	+	.	.	.	.	.	+	.	.	.	.	12	29	42	10	14	15
			BS	41	40	+	.	+	+	+	+	+	.	+	.	.	+	+	.	.	.	.	20			14		
		48	BS	30	29	+	+	.	+	+	+	+	.	.	+	.	+	+	.	.	.	.	17			11		
	43	24	SS	30	21	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	15	29		11	13	
			BS	34	32	+	+	+	+	+	+	+	.	+	.	.	.	.	.	.	.	.	18			12		
		48	BS	31	29	+	+	+	+	.	+	+	.	.	.	.	+	.	.	.	.	.	17			12		
III. Strontium chloride B	37	24	SS	39	11	+	+	+	.	.	.	+	.	.	+	.	.	.	.	.	.	.	8	35	60	7	16	16
			BS	40	33	+	+	+	+	+	.	.	+	+	.	.	+	.	.	.	.	.	19			14		
		48	BS	29	20	+	+	.	+	+	+	.	+	.	.	+	.	.	.	.	.	.	17			12		
	43	24	SS	33	27	+	+	+	+	+	+	+	.	.	+	+	.	.	.	.	.	.	20	47		14	15	
			BS	44	41	+	+	+	+	+	+	+	+	+	+	.	.	.	.	+	.	.	24			15		
		48	BS	36	35	+	+	+	+	+	+	+	.	.	+	+	.	.	.	.	.	+	24			15		
IV. Strontium chloride B with swab	43	24	SS	41	10	+	+	.	+	.	.	.	.	+	.	+	.	.	.	.	.	.	8	49		7	17	
			BS	50	48	+	+	+	+	+	+	+	+	+	.	+	.	.	.	.	.	.	35			17		
		48	BS	37	33	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	24			16		
Serum recovery technique	37	24	BS	77	72	+	+	+	+	+	+	+	.	+	.	.	.	+	.	.	.	.	23	57				
	43	24	BS	127	126	+	+	+	+	+	+	+	.	+	+	.	.	.	+	.	+	.	48					
Totals				966	744	15	13	11	9	7	9	4	5	9	3	3	2	1	3	1	1	1		97			18	

factory effluents. Differences in the performance of enrichment media were most evident with samples of abattoir effluent, where Rappaport and strontium chloride M broths were each considerably better than selenite F enrichment. However, the performance of selenite F was considerably improved and that of strontium chloride M broth further improved when effluent samples were incubated at 43° C. (Table 4). Rappaport broth was not incubated at the higher temperatures.

The *Salmonella* serotypes recovered from human faeces, meats and meat-processing centres during the food-poisoning investigations are listed in Table 5. *Arizona* 26:26–25 was also recovered from human faeces and *Arizona* 16:23–25 was isolated from a sample of kangaroo meat.

The results obtained in the third investigation, Study III, are set out in Tables 6–8. One hundred and five *Salmonella* isolations, comprising 19 serotypes, were made from 18 positive samples of sea water contaminated with abattoir effluent. At a temperature of 37° C. (Table 6) strontium chloride B enrichment was slightly better than either strontium selenite or Rappaport in the total number both of isolations and of individual serotype recoveries. However, the total of positive *samples* achieved by each method was fully comparable.

The performance of strontium chloride B and strontium selenite was enhanced when the enrichment media were incubated at 43° C. But Rappaport was more suited to the lower temperature of incubation. The distribution and relative frequency of *Salmonella* serotypes, isolated by the four enrichment methods subcultured at 24 and 48 hr. and by the supplementary sero-recovery technique, are shown in Table 6.

If a single enrichment broth, a single incubation temperature and a single plating medium are to be used, strontium chloride B medium when added to the swab sample and incubated at 43° C. with subculture after 24 hr. incubation to the modified BS agar, was the best single method consistent with economy of materials; 17 (94 %) positive samples, 10 (59 %) serotypes, and 35 (33 %) isolations were recorded; furthermore, only 50 (5·2 %) colonies were examined and of these 48 (96 %) were positive. There was little difference in the isolations and positive sample totals when strontium chloride B medium was used to examine either the fluid or swab sample by the elevated temperature technique. However, three more serotypes were isolated from the fluid sample than from the swab.

The *Salmonella* isolations, serotypes and positive samples recovered from enrichment media at 24, 48 and 72 hr. and without the use of the supplementary sero-recovery technique are detailed in Table 7. The combined 24 hr. subculture procedures recovered a total of 78 (80 %) isolations, 14 (74 %) serotypes and 18 (100 %) positive samples, compared with 64 (66%) isolations, 15 (79%) serotypes and 18 (100%) positive samples at 48 hr. The recoveries from the 14 samples subcultured to BS agar at 72 hr. were 38 isolations, 16 serotypes and 14 positive samples. The 48 hr. subculture routine increased the 24 hr. isolation total by 11 and the serotype numbers by 2, whereas the 72 hr. subculture increased the isolations by a further 8, and three additional serotypes *S. muenchen*, *S. oranienburg*, and *S. potsdam* were isolated. *S. muenchen* was also recovered by the 24 hr supplementary sero-recovery method (Table 6).

Table 7. Salmonella *isolations, serotypes and positive samples from* 18 *effluent samples examined by four enrichment methods, incubated at* 37 *and* 43° C. *and subcultured to* SS *agar and modified* BS *agar after* 24, 48 *and* 72 *hr.*

Subculture after (hr.)	Plating media	No. of *Salmonella* isolations at (° C.)			*Salmonella* serotypes at (° C.)			Positive samples at (° C.)		
		37	43	37/43	37	43	37/43	37	43	37/43
24	SS	19	39	46	9	10	11	13	17	17
	BS	38	57	68	11	12	14	16	17	18
	SS/BS	45	65	78	12	12	14	16	17	18
48	BS	32	55	64	11	14	15	15	17	18
24/48	SS/BS	52	80	89	13	15	16	16	17	18
*72	BS	19	29	38	11	13	16	11	13	14
Totals		56	85	97	15	17	19	16	17	18

* Results from 14 samples only.

Table 8. *Distribution and relative frequency of* Salmonella *serotypes in* 909 *colonies on subculture media related to time and temperature of incubation of enrichment culture*

		No. of positive colonies after								
		24 hr. at				48 hr. at		72 hr.* at		
		37° C. on		43° C. on		37 °C on	43 °C on	37 °C on	43 °C on	No. of isola-
Serotype	Pos. cols.	SS	BS	SS	BS	BS	BS	BS	BS	tions
livingstone	209	15	42	39	53	12	24	8	16	15
derby	78	1	7	8	19	8	27	3	5	13
bredeney	61	3	5	5	15	.	18	.	14	10
typhimurium	66	5	11	8	15	14	11	2	.	10
bovismorbificans	36	.	8	2	15	.	7	1	3	8
havana	61	2	6	11	14	5	8	6	9	8
give	61	2	6	2	11	8	10	14	8	7
adelaide	18	.	3	.	4	2	2	1	6	6
senftenberg	21	2	2	1	4	4	4	2	2	4
bahrenfeld	11	1	.	1	3	1	3	.	2	3
wandsbek	8	.	.	3	1	2	2	.	.	3
anatum	10	.	5	.	.	2	3	.	.	2
newington	11	.	.	.	.	.	1	5	5	2
bukavu	6	2	1	.	.	1	.	2	1	1
chester	3	.	.	.	.	.	2	.	1	1
hvittingfoss	2	.	.	.	2	.	.	.	.	1
muenchen	1	.	.	.	.	.	.	.	1	1
oranienburg	2	.	.	.	.	.	.	.	2	1
potsdam	1	.	.	.	.	.	.	1	.	1
Totals	666	33	96	80	156	59	122	45	75	97
Colony tests	909	106	128	144	170	80	134	62	85	.
% positive	73·3	31·1	75·0	55·6	91·8	73·8	91·0	72·6	88·2	.

* Results from 14 samples only.

The multi-serotype serum recovery method performed on the 24 hr positive BS agar plates increased the 24 hr BS isolation total from 68 to 83 and the serotype total from 14 to 16. The combined enrichment media isolation total was increased from 97 to 105. However, 42 isolations detected by the routine 24 hr. isolation procedures were again recovered by the serum method, and additional serotypes were subsequently detected in the 48 and 72 hr. subculture.

In the 24 hr. enrichment subculture routine, where both SS and BS agar media were used (Table 7) the modified BS agar was considerably better as a plating medium; for example, after enrichment at 37° C. only 19 *Salmonella* isolations, 9 serotypes and 13 positive samples were recovered from SS agar, compared with 38 isolations, 11 serotypes and 16 positive samples from BS agar. Salmonella isolations from SS plating media were increased from 19 to 39 when enrichment media were incubated at 43° C. Isolations from BS agar were increased from 38 to 57, when the higher enrichment temperature was used.

The 24 hr. isolation total from enrichment media plated out on modified BS agar was 68 isolations, 14 serotypes and 18 positive samples, compared with 46, 11 and 17 respectively from SS. agar. Furthermore, 252 (85 %) colonies were positive, compared with 113 (45 %) positive colonies from SS. However, the SS plating routine increased the BS agar isolation total from 68 to 78. The distribution and relative frequency of *Salmonella* serotypes in the 659 colonies selected from BS agar and in the 250 colonies examined from SS plating medium are detailed in Table 8.

DISCUSSION

In a report on salmonellosis in Australia, Atkinson (1964) commented that the epidemiology of the disease was not well understood, few outbreaks had been closely studied and individual cases seldom followed up. Nevertheless, *Salmonella typhimurium*, *S. derby*, *S. anatum* and *S. adelaide* were noted as widespread pathogens, passing between man, animals and the environment with considerable efficiency. *S. typhimurium* was involved more than any other serotype, but an outbreak of *S. derby* infection was reported by Rubbo (1948); and Lee & Mackerras (1955) reported a severe outbreak of gastro-enteritis due to *S. bovismorbificans*.

In the present investigations *S. typhimurium* was directly implicated in the food-poisoning outbreak and this serotype, as well as *S. derby*, *S. anatum*, *S. adelaide* and *S. bovismorbificans*, was frequently detected in samples from domestic animals, abattoirs and meat-processing effluents. *S. typhimurium* was rarely found in kangaroo meat, domestic pets and reptiles. *S. muenchen* was widely distributed, particularly in kangaroo meat and rodents. *S. rubislaw* was recovered exclusively from human specimens during the food-poisoning investigations, but this serotype has been isolated from cattle and rodents in Australia (Atkinson, 1964) and from lizards (Lee & Mackerras, 1955; Iveson, Mackay-Scollay & Bamford, 1969).

S. typhimurium was isolated from 86 of the 97 patients (172 isolations) diagnosed during the outbreak. The source of infection was traced to a meat smallgoods factory, and samples of corned beef, ham, ham salami, polony, saveloys, frankfurts,

and swabs from shelves, benches, floor, tables, mincer, meat pack sealing machine, delivery truck, refrigerated store and sewage, also yielded *S. typhimurium*. Salmonella strains from meat products, eight food handlers, and from human cases in different suburbs were identified as *S. typhimurium* phage type 21.

With the exception of *S. rubislaw*, the serotypes recovered from patients and contacts during the food-poisoning investigations were all recovered from livestock, abattoir effluents and meat processing centres. The isolation of *S. chester* and *S. havana* were of particular interest as both serotypes were subsequently associated with outbreaks of human infection. The *S. havana* outbreak was traced to infected food handlers and meat products. The serotype was also recovered frequently from livestock at the abattoir supplying the meat centre. The prevalence of a serotype in an abattoir followed subsequently by human infection has been reported by Harvey & Price (1970).

With the exception of *S. pullorum*, *S. houten* and *S. waycross*, the 39 *Salmonella* serotypes recovered from livestock, domestic pets, rodents, abattoirs, meats and meat processing centres have all been isolated from cases of human infection in Western Australia. A total of 18 (72 %) serotypes recovered from reptiles, have also been isolated from man. Seven serotypes, *S. boecker*, *S. bootle*, *S. bullbay*, *S. kibusi*, *S. kisarawe*, *S. sydney* and *S.* 42 : z : –, have been recovered exclusively from reptiles. The public health significance of salmonella carriers in livestock and birds has been reported by Hobbs (1961).

The detection of a single salmonella serotype in a contaminated sample, whilst important from a diagnostic public health viewpoint, does not necessarily provide accurate epidemiological data, particularly in the examination of foodstuffs and environmental samples. The problems of assessing the diagnostic and epidemiological significance of multiple serotype infections has been reported by Winkle & Rohde (1958), and, more recently, Harvey & Price (1967) have drawn attention to the technical problems associated with the isolation of multiple serotypes from heavily contaminated materials, and have commented further that the choice of technique depends on the time available for the examination and the epidemiological importance of achieving comprehensive results. A wide choice of isolation techniques are available and, in comparative studies, the results obtained by different laboratories have shown wide variation (Edel & Kampelmacher, 1968, 1969). The importance of the sampling and salmonella isolation procedure in relation to the final result has also been stressed by Van Schothorst & Kampelmacher (1967). They reported that isolations in their laboratory varied from 28 to 57 % according to the method of examination.

In the present study in tests on domestic animals, rodents and reptiles, the inclusion of strontium chloride M and Rappaport broth, along with the direct culture combined with selenite F routine, increased salmonella isolations from 159 to 234 (47 %), and 159 to 219 (38 %) respectively. The combined increase was 159 to 280 (76 %). Reduced selenite performance was most evident with faeces inocula from pigs, cats, dogs and rats, where *Proteus* species were frequently encountered. The problems of *Proteus* overgrowth with selenite F and tetrathionate media has been reported by Hobbs & Allison (1945), Smith & Buxton (1951),

Cruickshank & Smith (1949), and Galton, Scatterday & Hardy (1952). In the animal studies both Rappaport and strontium chloride M were effective in suppressing the growth of *Proteus* species, and salmonellas were often isolated by both procedures when the corresponding selenite F subculture showed a heavy growth of *Proteus* or, occasionally, *Pseudomonas* species. Using laboratory strains, Anderson & Kennedy (1965), and Vassiliadis (1968), also showed that Rappaport broth was more efficient than selenite F or tetrathionate media in suppressing *Proteus* species, and at the same time allowing satisfactory growth of *S. choleraesuis*.

However, in contrast to the results of the present investigation (cf. Table 2), the latter author found selenite F broth superior to Rappaport enrichment medium in a growth test series in which *Arizona* strains were used.

In tests on human faeces Chau & Huang (1971) reported that strontium selenite broth was superior to selenite F, both in the inhibition of *Proteus*, and in the isolation of *S. typhi*. Strontium chloride M and Rappaport broth were found suitable for the isolation of *S. choleraesuis*. The latter methods were also effective in the recovery of the selenite-sensitive serotype from pig glands in the present investigation.

The strontium chloride M and Rappaport media were slightly better than selenite F in tests on human faeces, meat products and effluent samples examined in the food-poisoning investigations reported here. However, when effluent samples were incubated at 43° C., the performance of selenite F was improved considerably, although it was still inferior to enrichment in strontium chloride M.

Salmonella organisms were detected in each of the 70 abattoir effluent samples examined, whether diluted with sea water (Study III) or not, and a total of 226 isolations, which comprised 24 serotypes, were achieved. In the final study in which strontium selenite, strontium chloride B and Rappaport enrichment were combined with the serum recovery method, 105 salmonella isolations were obtained from 18 sea-water samples contaminated with abattoir effluent. One sample yielded 10 *Salmonella* serotypes, and a total of 19 serotypes were identified in specimens collected over a 24 hr period.

The value of a comprehensive testing programme in achieving both diagnostic and epidemiological data, together with the work load involved, has been amply demonstrated in the present investigation. It has also been shown, particularly in tests on abattoir effluents reported in Table 6, that the new strontium chloride and strontium selenite enrichment media are fully capable of achieving comprehensive results when used singly or in combination. The choice of a single enrichment broth, suitable plating medium, and number of colonies selected for identification, will be governed by both epidemiological requirements and the resources of the laboratory. The new media have shown that they can perform efficiently when incubated at 43° C., and provide a sound basis for the application of the supplementary serum recovery method to the single 24 hr. modified BS agar subculture routine for the isolation of multiple serotypes.

Throughout the investigations, the modified bismuth sulphite agar plating medium was greatly superior to SS agar and provided ready differentiation of *Salmonella* and *Arizona* species within 24 hr. incubation.

We wish to express our thanks to Dr Joan Taylor for confirming the identity of *Salmonella* serotypes, to the late Dr K. Patricia Carpenter for confirming the *Edwardsiella* strain, to Dr R. Rohde for serotyping many of the *Arizona* strains, and to Dr E. S. Anderson for kindly phage typing cultures of *S. typhimurium*. We are greatly indebted to Mr M. H. Lello and the Ajax Chemical Company for undertaking the manufacture of strontium hydrogen selenite salt; to Mr V. Bamford and the laboratory team; to Dr K. Black, Mr H. Pickett and Mr J. Wallis of the Perth City Health Department, and to Dr R. Lugg of the Department of Health, Western Australia.

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Corrigenda

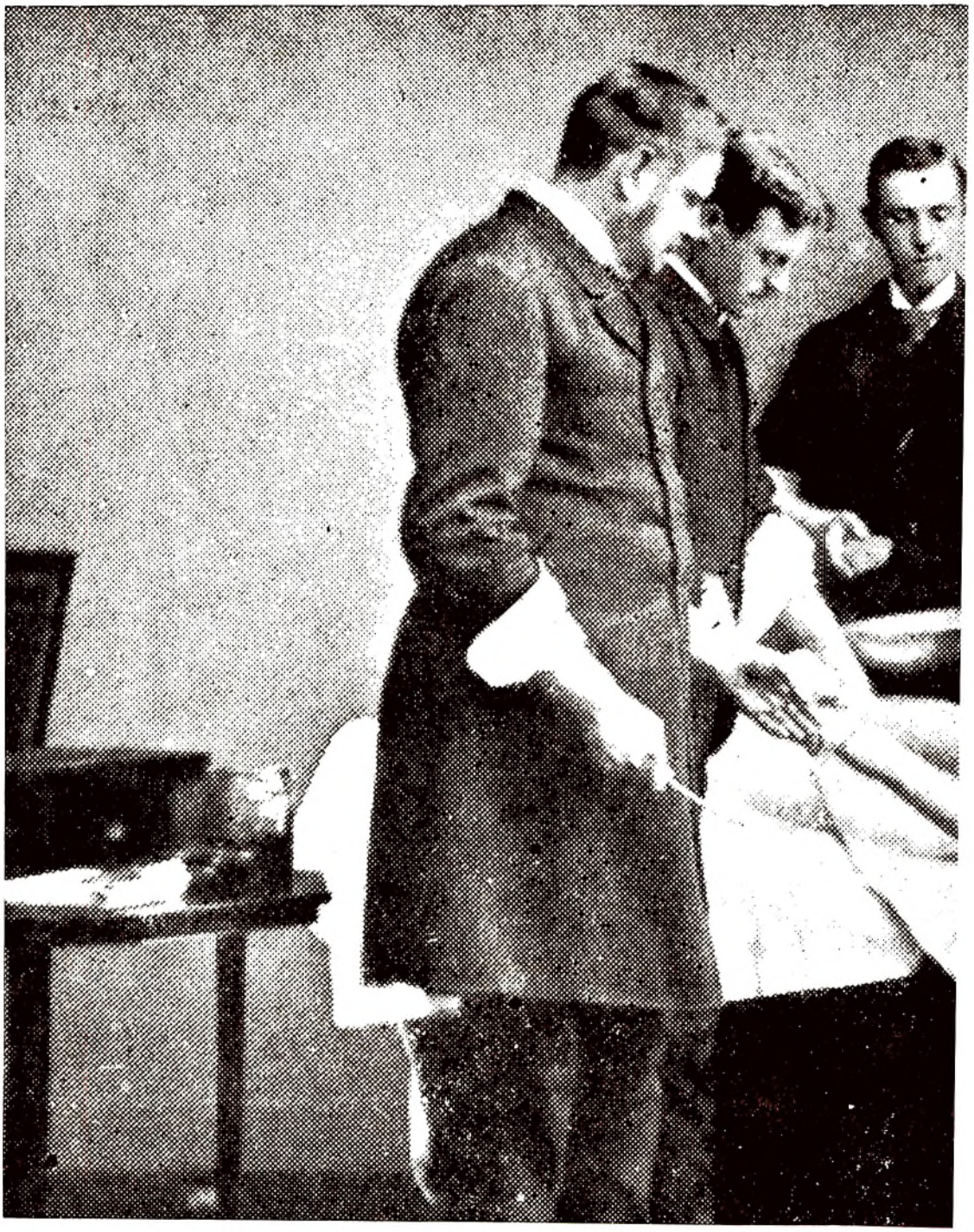
Public Health Laboratory Service Working Group, NIELS SKOVGAARD and B. BREST NIELSEN. *Journal of Hygiene* (1972) **70**, 127–40.

On page 132, legend to Table 4 should read *Growth of* Salmonella *from various raw materials sampled from November* 1968 *to January* 1970 *in one factory, and which can be used in the manufacture of pig feed.*

A. C. GHOSH. *Journal of Hygiene* (1972) **70**, 151–60

On page 154, in footnote to Table 1;

For R = *S. bredeney read* B = *S. bredeney*



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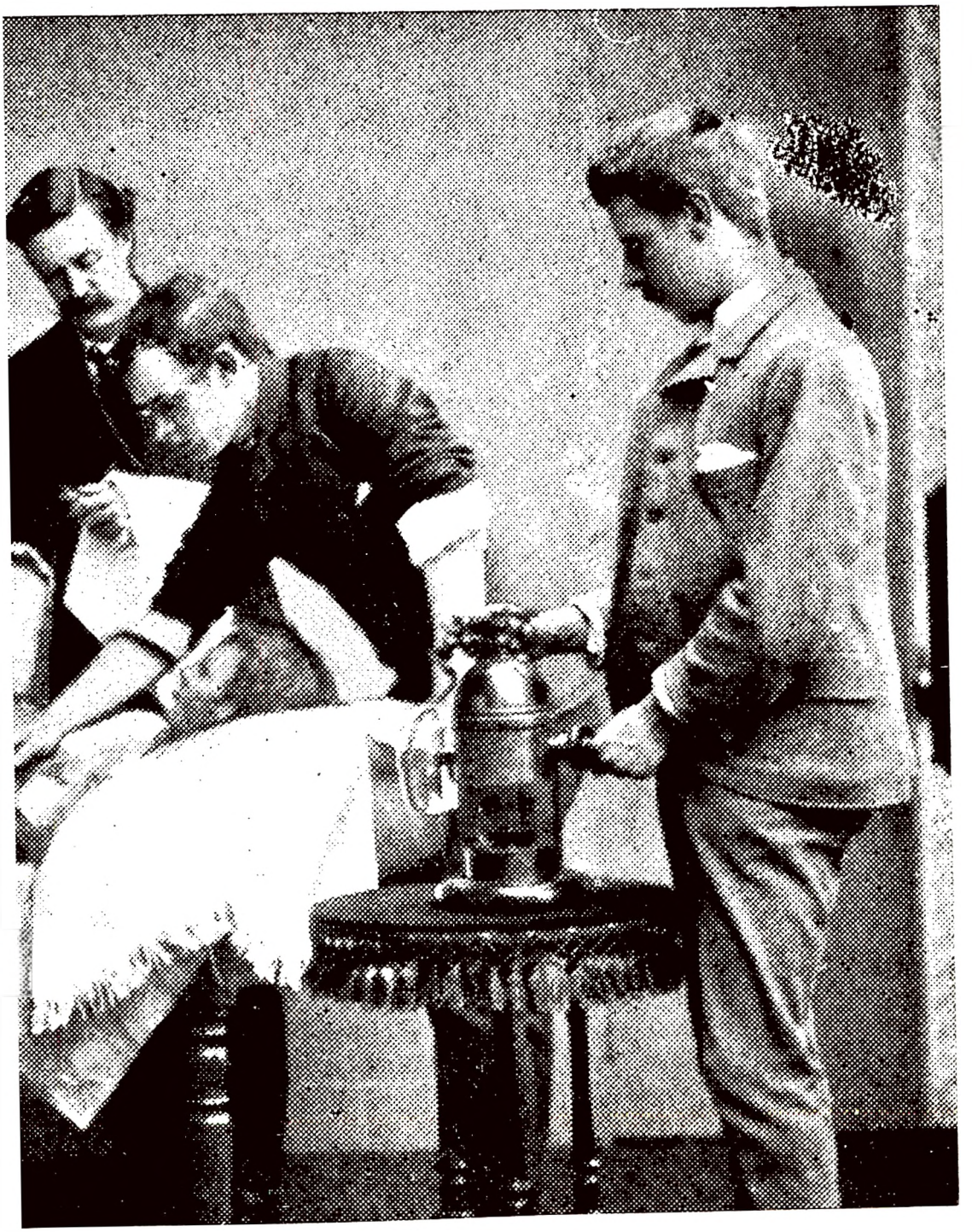

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