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The size distribution of airborne particles carrying micro-organisms

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The ability of a particle to remain airborne, its ability to pass through filters, the site at which it may be deposited in the respiratory tract and the rate at which it will be removed from the air by sedimentation are all dependent on the size and density of the particle. In the course of a variety of investigations we have determined the size distribution of particles carrying various species of bacteria and fungi, using the size-grading slit-sampler described by Lidwell (1959). A few of the results obtained have already been quoted in part, but the majority have not been published previously.

The air sampler used separates the airborne particles into four size ranges, each of which is deposited on the surface of the agar medium contained in one of four 6 in. Petri dishes. This apparatus is constructed so that the air sample, entering through a slit 7 mm. wide, impinges on to the surface of the first Petri dish at such a velocity that only the larger particles, i.e. those having an equivalent particle diameter† greater than about 18μ, are deposited. The air stream carrying the smaller particles is then caused to impinge in turn on to the surface of the three remaining Petri dishes, each time at an increased velocity and through a narrower slit, so that the minimum particle size for 50% deposition is about 10μ for the second dish, 4μ for the third and less than 1μ for the fourth and last. When the plates have been incubated the colonies found will be derived from organisms which entered the sampler carried on airborne particles corresponding approximately to the four size ranges, greater than 18μ, between 18 and 10μ, between 10 and 4μ and less than 4μ. These size limits correspond to the value of equivalent particle diameter for 50% deposition so that there is, in fact, a considerable size overlap between the fractions. In spite of this, however, reasonably good estimations of the particle size distribution within the sample and hence of the median equivalent particle diameter and of the dispersion, expressed either as an interquartile range or, if appropriate, as a standard deviation, can be made by plotting on probability paper the cumulative fraction oversize against the 50% collection limits, namely 18·2, 9·6 and 4·2μ. As there are internal losses in the instrument it is necessary to correct the numbers of colonies counted in the later stages in order to arrive at a good estimate of the size distribution in the original sample. The numbers found on

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† The equivalent particle diameter is the diameter of a sphere of unit density which has a settling rate in air equal to that of the particle in question.

plates 2, 3 and 4 should be multiplied by 1·10, 1·20 and 1·25, respectively. These factors are derived from experimental observations.

CULTURAL METHODS

Samples for *Staphylococcus aureus* were collected on nutrient agar containing 5 % horse serum and 0·1 % phenolphthalein-phosphate (Barber & Kuper, 1951). Phosphatase positive colonies were tested for coagulase. Counts of total flora and of aerobic spore-bearing bacilli were made on the same medium but the phenolphthalein-phosphate was sometimes omitted.

Streptococci were grown on serum-sucrose agar containing tellurite and crystal violet (Williams & Hirch, 1950). The levan-producing colonies of *Str. salivarius* (Williams, 1956) were recognized by their colonial appearance and counted after 40–48 hr. incubation at 37° C. The other streptococci and the enterococci were estimated by picking a random sample of colonies from these plates and examining them by methods previously described (Air Hygiene Committee, 1954, Routine C, pp. 52–4).

Samples for *Clostridium welchii* were collected on a modified Nagler medium containing neomycin. Those colonies showing zones of serum opacity were regarded as *Cl. welchii* (Noble, 1961).

Sabouraud's dextrose agar containing antibiotics to suppress bacterial growth was used for isolating fungi. Cultures were incubated for 3 days at 37° C. for the *Aspergilli* and the *Candida* species and up to 3 weeks at room temperature for the remaining fungi. When sampling for *Candida albicans* or the dermatophyte fungi, Actidione (0·5 g./l.) was added to the medium.

RESULTS

The values deduced for the median equivalent particle diameters and for the inter-quartile ranges are given in Tables 1 and 2. These were obtained in the way described previously (Lidwell, 1959). Estimates of the inter-quartile ranges, the limiting diameters defining the 25 % smallest and the 25 % largest particles, are given, rather than standard deviations of the diameter, since the forms of the distributions are not known. In most cases an arithmetic-normal distribution appeared to fit the data reasonably well, where the median equivalent particle diameter was greater than 10μ; if the median diameter was smaller than this a log-normal distribution usually appeared the better. We have, however, no information about the tails of the distribution except that these must be truncated at or above about 1μ for the bacteria and at sizes corresponding to that of the single cells for the fungal species. Some environmental factors can be seen to affect the recorded values of median equivalent particle diameter. Ventilation preferentially removes the smaller particles so that the median diameter normally becomes greater when the ventilation is increased. The staphylococcal samples show a small but definite increase in median diameter with increasing activity during the sampling period. A similar, and partly associated, variation with the amount of air contamination is illustrated in Fig. 1.

Table 1. *Airborne bacteria*

Species	Place	Activity, etc.	Colonies counted	Median equivalent diameter of the airborne particles (μ)	Inter-quartile range (μ)
Total aerobic flora (grown at 37° C.)	Offices	Low ventilation	> 15,000	7·7	4–11
	Offices	Good ventilation	> 8,000	10·0	5–15
	Hospital wards	Moderate	> 50,000	12·8	7–18
	Hospital wards	Considerable	> 30,000	13·0	8–18
	Operating rooms	Unoccupied	> 30,000	12·3	7–18
Total mouth streptococci	Offices	Low ventilation	> 800	10·0	4–16
	Offices	Good ventilation	> 300	12·4	6–18
Streptococcus salivarius	Offices	Low ventilation	> 500	11·0	4–18
	Offices	Good ventilation	89	14·4	7–(22)
Beta-haemolytic streptococci	Offices	Low ventilation	29	11·7	8–15
	Offices	Good ventilation	22	12·5	8·5–16·5
Enterococci	Offices	Low ventilation	83	11·0	6–16
	Offices	Good ventilation	50	10·8	4–17
Staphylococcus aureus	Hospital wards	Light–moderate	> 6,000	13·3	8–18
	Hospital wards	Bedding disturbed	> 7,000	14·8	10–(19)
	Hospital wards	Bed-making	> 2,000	15·7	11–(20)
Bacillus sp.	Hospital ward	Moderate	> 300	(3·0)	(?)–8
Clostridium welchii	Outside air	Wet weather	186	11·0	5·5–16·5
	Outside air	Dry weather	> 500	17·2	10–(24)
	Hospital wards	Moderate (wet weather)	299	11·4	4–18

The inter-quartile range is given as the limiting diameters defining the 25% smallest and the 25% largest particles. Where the estimated diameters are below 4μ or above 18μ the values depend on extrapolation and have been given in brackets to indicate the greater possibility of error.

The samples of air from the offices were obtained in several different rooms of a large group of offices over a period of months.

The hospital samples came from a number of different hospitals.

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Table 2. *Airborne fungi*

Species	Place and activity, etc.	Colonies counted	Diameter of spore or single cell (μ)	Median equivalent diameter of the airborne particles (μ)	Inter-quartile range (μ)
*Aspergillus fumigatus**	Hospital ward, moderate	> 100,000	2·5–3·5	(3·0)	[3–3]
Penicillium spp.	Hospital ward, moderate	> 750	2·5–4·5	(3·1)	[2–4]
Paecilomyces spp.	Hospital ward, moderate	105	(2·5–3) × 6	(3·4)	(2)–6
Rhodotorula spp.	Hospital ward, moderate	> 4,000	(3–5) × (4–7)	(3·8)	[3·3–4·3]
Aspergillus spp.†	Hospital ward, moderate	159	2·5–4	4·3	(2)–8
Cladosporium spp.	Hospital ward, moderate	> 2,500	(2–6) × (3–20)	4·9	(3)–7
Aspergillus niger	Hospital ward, moderate	125	2·5–10	5·5	(3)–9
Syncephalastrum spp.	Hospital ward, moderate	240	(2·5–5) × (5–20)	6·6	4–10
Rhizopus spp.	Hospital ward, moderate	45	3 × (6–9)	6·8	4–10
Monilia sitophila	Hospital ward, moderate	74	3 × (3–10)	9·5	7–13
Didymocladium spp.	Hospital ward, moderate	> 500	5 × (10–15)	10·7	7–15
Candida albicans	Hospital ward, moderate	67	(3–6) × (3–12)	13	5–(21)
Trichophyton mentagrophytes‡,§	Clinic, scraping and examination of skin	> 300	2 × 4 (*a*) (4–6) × (10–50) (*b*)	16	11–(22)
Rhodotorula spp.‖	Clinic, scraping and examination of skin	118	(3–5) × (4–7)	18	11–(26)
Epidermophyton floccosum§	College dormitories, little	18	(6–10) × (20–30)	19	12–(26)
E. floccosum	Clinic, scraping and examination of skin	172	(6–10) × 20–30)	(21)	15–(26)
Candida albicans	Clinic, scraping and examination of skin	> 1,000	(3–6) × (3–12)	(22)	18–(27)

The inter-quartile range is given as the limiting diameters defining the 25% smallest and the 25% largest particles. Where the estimated diameters are below 4μ or above 18μ the values depend on extrapolation and have been given in brackets to indicate the greater possibility of error involved. Substantial correction has had to be applied to the values of the quartiles for *Asp. fumigatus*, *Penicillium* spp. and *Rhodotorula* spp., found in hospital wards, on account of the diffuse cut-off of the stages in the instrument, and these are enclosed in square brackets. Where the spore or cell concerned is markedly non-spherical the approximate ranges of both the shortest and the largest axes are given in brackets, linked by a multiplication sign. In the case of *Trichophyton mentagrophytes*, (*a*) refers to the microconidia and (*b*) to the macroconidia.

* In seven separate evaluations the estimated median equivalent diameters ranged from 1·8 to 4·1 with an indication that the value tended to be larger in the winter.

† Including *Asp. gracilis*, *Asp. nidulans*, *Asp. terreus* and *Asp. versicolor*.

‡ This was derived from examination of an artificially contaminated arm.

§ Spores of these organisms were never seen in material obtained directly from patients. Only mycelium could be seen in the infected skins.

‖ This organism is not known to have been associated with any patient.

The hospital ward samples were obtained in various places on different occasions.

In comparing the figures for median equivalent particle diameter given here with those deduced from the ratio between volumetric and settling counts (Bourdillon, Lidwell & Lovelock, 1948) it must be remembered that these latter are primarily estimates of the mean settling rate, i.e. of the mean-square diameter. The relation between this quantity and the median diameter as determined here or by data

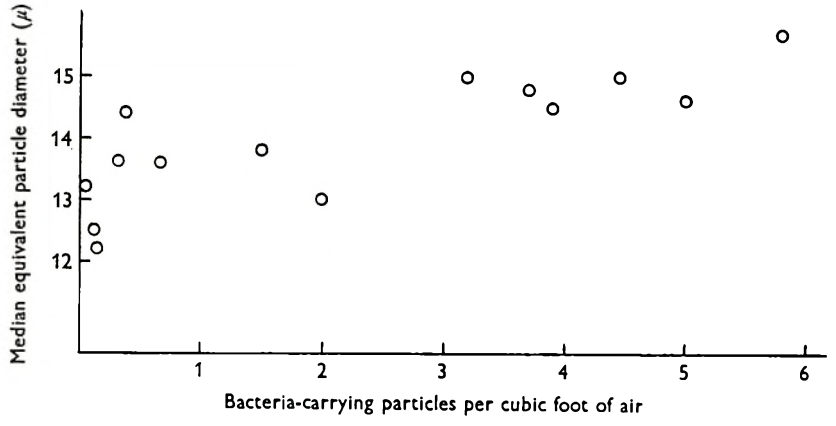

Fig. 1. Median diameters of airborne particles carrying *Staphylococcus aureus*.

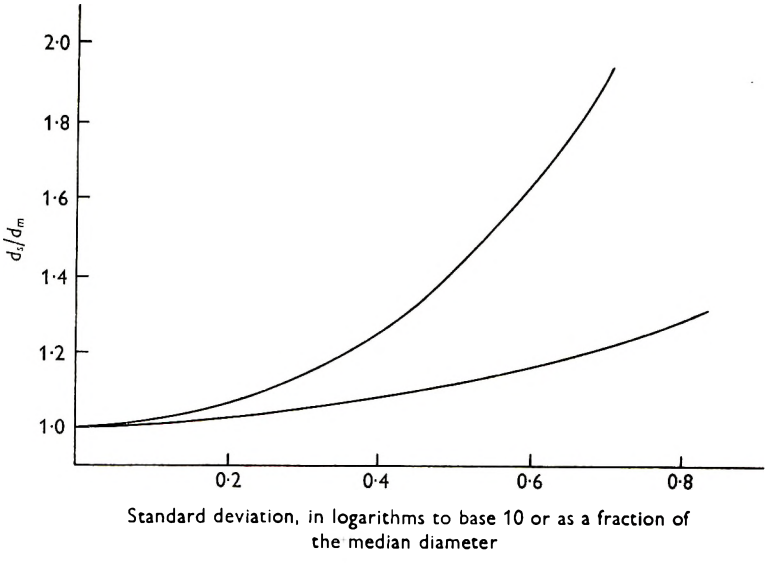

Fig. 2. Relationship between mean settling diameter, d_s, and median diameter, d_m. The upper of the two curves refers to log-normal distributions. The arithmetic-normal distributions, lower curve, are truncated at zero diameter and their standard deviations are expressed as a fraction of the median diameter.

obtained in a similar way, e.g. samples taken with the Andersen or Batelle samplers (Andersen, 1958; Mitchell & Pilcher, 1959), depends on the distribution of particle size in the sample. Fig. 2 gives values for the ratio of the two quantities for both arithmetic-normal and log-normal distributions over a range of values of the standard deviations of these distributions.

The following example illustrates these points.

At the same time as the samples of total flora were collected in the operating theatres with the size-grading sampler (see Table 1), settling plates were also exposed. The mean rate of settling over the whole series of experiments was 5·89 colony-forming particles per minute per square foot of surface exposed. The mean volumetric count over the same period was 4·90 cu.ft. of air sampled. This corresponds to a mean settling rate for the particles concerned of 5·89/4·90 = 1·20 ft./min. Using the figure of $0{\cdot}006d^2$ for the settling rate in feet per minute of a unit density particle d microns in diameter this corresponds to a mean equivalent settling diameter of $(1{\cdot}20/0{\cdot}006)^{\frac{1}{2}} = 14{\cdot}2\mu$.

The median equivalent diameter of the particles as determined by the size-grading sampler was $12{\cdot}3\mu$ and the distribution conformed well to a normal distribution in the arithmetic value of the diameter with a standard deviation of $8{\cdot}0\mu$. The ratio of this standard deviation to the median diameter is 0·65 and from the lower curve in Fig. 2 this would correspond to a median settling diameter 1·18 times the median diameter, i.e. $12{\cdot}3 \times 1{\cdot}18 = 14{\cdot}5\mu$, which agrees closely with the estimate obtained above by direct comparison of the settling and the volumetric counts, namely $14{\cdot}2\mu$.

The estimated standard deviations of the size distributions of the airborne bacteria-carrying particles which we have so far examined have almost always fallen within a fairly narrow range, about 0·3 for a log-normal distribution to the base 10 or about 0·6 times the median diameter for arithmetic-normal distributions. For standard deviations of this magnitude the mean settling diameter is about 15% greater than the median diameter and this value may be used when comparing particle diameters estimated by the two methods where detailed information on the size distributions are not available.

DISCUSSION

The most striking fact revealed by these data is the size of the median equivalent diameter associated with almost all the bacterial species and with those fungi that are probably derived from a human source, that is, the dermatophyte species, *Trichophyton* spp. and *Epidermophyton* spp. and *Candida albicans*. These diameters, which are much greater than the dimensions of the microbial cell, imply that the organisms are usually disseminated into the air in association with material derived either from the menstruum in which they originally multiplied or from some intermediate resting place. There is some evidence (Williams, Lidwell & Hirch, 1956) that the streptococci are derived directly from the mouth and that the particle therefore largely consists of dried saliva. Davies & Noble (1962) have recently presented evidence that many, if not most, staphylococci are present in the air attached to skin scales which could account for the observed diameters. It also seems likely that infected skin scales are responsible for the dissemination of the dermatophyte fungi. The size distribution of airborne particles carrying micro-organisms is determined by two opposing factors, gravity, which tends to eliminate the large particles, and the chance that a particle will carry a viable organism, which is likely to increase with the size of the particle. These factors combine to

confine the distribution within a relatively narrow range over the main part of which, at any rate, the distribution approximates closely to the arithmetic normal.

In contrast many fungi such as the aspergilli, penicillia, cladosporia, among those included in the observations reported here, possess a mechanism for direct dispersal of their spores into the atmosphere from their natural growth sites. This is reflected in the close correspondence for many of these species between the median equivalent particle diameters found for the air sample and the size of a single spore. Within the observed portion these distributions approximate to a log-normal form.

The most marked exception to the general pattern described above is the particle diameter associated with the aerobic spore-bearing bacilli. This is not much larger than the single cell although there is a very wide spread of particle size. It is possible that this is a result of the capacity of these organisms to multiply in extremely dilute media, in which sporulation commonly occurs, so that when the spores are dispersed by mechanical action they are only very loosely bound together and embedded in only small amounts of dried material.

SUMMARY

Values are given for the median equivalent diameters and for the inter-quartile range, of airborne particles carrying a variety of micro-organisms.

Organisms associated with human disease or carriage were usually found on particles in the range 4–20μ equivalent diameter.

Many fungi appeared to be present in the air as single spores.

We should like to acknowledge the collaboration of Dr Yvonne M. Clayton in the collection and examination of the fungal samples.

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Cleaning of hospital floors with oiled mops

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Large numbers of bacteria are dispersed into the air when untreated floors are swept with a broom. Contamination from this source has been controlled by treating floors with spindle oil (van den Ende, Lush & Edward, 1945), but the method has some drawbacks, in particular the damage that it causes to leather shoes. An effective alternative to oiling of floors which is free from these drawbacks is the use of vacuum cleaners with adequate filters (Rogers, 1951; Bate, 1961). Another method by which the dispersal of floor dust may be reduced is the use of impregnated mops (Burnham, 1962). We describe here a comparative study of floor cleaning in a hospital ward by mops impregnated with a mixture of oils, by a vacuum cleaner and by brooms; the methods were compared in respect of their removal of bacteria from the floor and the contamination of air during their use.

MATERIALS AND METHODS

Floor-cleaning equipment

The special mops ('Kex') (see Pl. 1) had been treated with a mixture containing mineral and vegetable oils and other ingredients. It was claimed by the manufacturers that the mops would absorb dust and prevent its dispersal into the air. These properties would be retained on storage for about 2 weeks, after which it was recommended that the treatment with oils should be repeated; for this purpose a service of fortnightly collection and delivery had been established, ward mops being replaced from the hospital store every 2 or 3 days. During the experiments described here a supply of treated mops was delivered 2 or 3 days before they were used for sweeping a ward, and collected at the time of delivery of freshly treated mops for the next experiment. A special handle and frame for the mops was provided by the manufacturer.

The vacuum cleaner used in these studies was an 'Electrolux' Model 33, with an air filter attached to the outlet of the tank container.

Sampling of floors for bacteria

Impression plates were taken from selected areas of floor by a method described by Foster (1960). Strips of sterile bandage, $1\frac{1}{2}$ in. wide, were laid across the bottom of sterile Petri dishes before a nutrient agar medium was poured into them. Samples were taken by removing the disk of medium, with the aid of forceps, by the projecting ends of the bandage; the outer surface of the Petri dish was applied over the medium, and with it the medium was pressed gently but firmly for

15 sec. over the selected area of floor. The agar disk was then transferred to the lid of the Petri dish, and incubated at 37° C. for 24 hr. In most of these experiments the medium used was phenolphthalein diphosphate agar (with 4% New Zealand agar), which allowed a presumptive count of *Staphylococcus aureus* as well as total viable counts (Barber & Kuper, 1951); in other experiments horse blood agar with 4% New Zealand agar was used. Surface viable counts were made with the aid of a hand lens.

Air sampling for bacteria

The air of the ward was tested with a large slit sampler, 11·5 cu.ft. of air being sampled on phenolphthalein diphosphate agar or horse blood agar.

Comparison of three methods of floor cleaning

Two open wards with wooden floors were used for the experiment. In one of the wards (I) the floor had not been treated with any oil for 6 months. In the other ward (II) the floor (2175 sq.ft.) had been treated 1 week before the first of three weekly experiments with 500 ml. of a mixture of linseed oil (1 part) and 'O'Cedar' oil (2 parts). The oil was applied with a polishing machine to improve the appearance of the floor, and was not expected to have any dust-laying properties; our tests confirmed this supposition.

In the first part of the experiment weekly tests were made in ward I; no beds were made during the hour before the test and while the test was in progress. Three tests were made with each of the three floor-cleaning methods, fresh oiled mops, vacuum cleaner and brooms; the three methods were used in rotation (i.e. a different method was used each week). Eight marked areas of floor in the middle of the ward were sampled before and immediately after the whole area of the floor had been cleaned. Air samples were taken before, during and immediately after cleaning. The time taken in cleaning the floor was noted.

In the second part of the experiment three further tests were made with each of the three cleaning methods. This time the oiled mops were tested when fresh and again 2 days later during their seventh use for cleaning the floor of ward I. Three further tests were made with a broom in ward II, and three further tests with the vacuum cleaner, two of them in ward I and one in ward II. Bacterial samples were taken as in the first part of the experiment.

RESULTS

Floor sampling

The bacterial counts of impression plates (mean of eight plates in each experiment) taken before and after floor cleaning are shown in Table 1. In each experiment the greatest reduction was found after the use of oiled mops; results were approximately the same with fresh oiled mops and with oiled mops used seven times. Bacterial counts were not reduced after the use of a broom. There was a significant difference in the mean percentages of initial counts in the comparison of the broom and the oiled mop ($t = 4{\cdot}67$, $P < 0{\cdot}001$), and in the comparison of

the broom and the vacuum cleaner ($t = 3{\cdot}33$, $P < 0{\cdot}01$). Presumptive counts of *Staph. aureus* (Table 3) showed a reduction after cleaning with the vacuum cleaner and the oiled mop, but a slight increase after the use of a broom; the numbers were small, and differences of means did not reach the level of statistical significance.

Table 1. *Removal of bacteria from floor by alternative cleaning methods*

			Mean viable counts of floor samples		
				After cleaning	
Cleaning method	Exp.	Ward	Before cleaning (initial count)	No.	% of initial count
Broom	1	I	1003	798	79·6
	2	I	773	579	74·9
	3	I	522	657	125·9
	4	II	524	547	104·4
	5	II	316	270	85·4
	6	II	456	448	98·2
	Mean	—	599·0	549·8	94·61 ± 7·7
Oiled mop (fresh)	1	I	387	119	30·7
	2	I	539	271	50·3
	3	I	864	300	34·7
	4	I	462	322	69·7
	5	I	394	224	56·8
	6	I	473	252	53·3
	Mean	—	519·8	248·0	49·3 ± 5·9
Oiled mop (used 7 times)	1	I	286	126	44·1
	2	I	303	163	53·8
	3	I	349	188	53·8
	Mean	—	312·6	159·0	50·5 ± 3·3
Vacuum cleaner	1	I	500	198	39·6
	2	I	547	287	52·5
	3	I	546	305	55·9
	4	I	669	486	72·6
	5	I	821	715	87·1
	6	II	848	464	54·7
	Mean	—	655·2	409·2	60·4 ± 6·8

Air sampling

The results of total counts are summarized in Table 2. They show a consistent rise (approximately a doubling) of airborne bacteria during the use of a broom, and a persistence of the raised air flora after the period of sweeping. By contrast, there was no increase (sometimes a fall) of airborne bacteria during the use of the vacuum cleaner, and after cleaning a consistent fall to a lower level than that found before cleaning. The oiled mop caused a small increase in airborne bacteria when used for the first time, but counts fell after cleaning to the numbers present at the beginning of the test. There was a significant difference in mean percentages of initial counts in the comparison of brooms with fresh oiled mops (during cleaning: $t = 3{\cdot}71$, $P < 0{\cdot}01$; after cleaning: $t = 4{\cdot}55$, $P < 0{\cdot}01$); also between the broom and the

vacuum cleaner (during cleaning: $t = 6{\cdot}80$, $P < 0{\cdot}001$; after cleaning: $t = 8{\cdot}82$, $P < 0{\cdot}001$) and between fresh oiled mops and vacuum cleaner (during cleaning: $t = 2{\cdot}51$, $P < 0{\cdot}05$; after cleaning: $t = 3{\cdot}71$, $P < 0{\cdot}01$). In the comparison of oiled mops used seven times with brooms there was no significant difference in tests made during cleaning, but the differences in counts obtained after cleaning were significant ($t = 3{\cdot}16$, $P < 0{\cdot}02$). When the mops had been used seven times they caused almost as much contamination of the air during sweeping as the broom, but these contaminants (unlike those dispersed by sweeping with a broom) fell rapidly from the air.

Table 2. *Contamination of air by alternative floor-cleaning methods*

		Viable counts of bacteria per cu.ft. of air				
			During cleaning		After cleaning	
Cleaning method	Exp.	Before cleaning (initial count)	No.	% of initial count	No.	% of initial count
Broom	1	124	270	217·7	270	217·7
	2	38	62	163·2	80	210·5
	3	67	134	200·0	124	185·1
	4	116	247	212·9	227	195·7
	5	82	168	204·9	190	231·7
	6	65	101	155·4	82	126·1
	Mean	82·0	163·7	192·3 ± 10·8	162·2	194·0 ± 15·2
Oiled mop (fresh)	1	51	49	96·1	51	100·0
	2	28	31	110·7	—	—
	3	143	169	118·2	103	72·0
	4	186	226	121·5	183	98·4
	5	68	131	192·6	107	157·3
	6	116	147	126·7	100	86·2
	Mean	98·7	125·5	127·6 ± 13·7	108·8	102·8 ± 14·5
Oiled mop (used 7 times)	1	137	243	177·4	152	110·9
	2	75	131	174·7	79	105·3
	3	48	105	218·7	69	143·7
	Mean	86·7	159·7	190·3 ± 14·3	100·0	120·0 ± 12·0
Vacuum cleaner	1	16	16	100·0	7	43·7
	2	237	293	123·6	149	62·9
	3	140	136	97·1	77	55·0
	4	76	40	52·6	38	50·0
	5	69	34	49·3	40	58·0
	6	85	58	68·2	14	16·5
	Mean	103·8	96·2	81·8 ± 12·1	54·2	47·7 ± 6·8

Although the differences in counts of presumptive staphylococci in the air did not reach the levels of statistical significance, they showed changes in relation to floor-cleaning methods similar to those shown by counts of total bacteria (see Table 3).

Table 3. *Presumptive* Staphylococcus aureus *removed from floor and dispersed into air*

Cleaning method	No. of experiments	Mean viable counts in floor samples			Mean viable counts per cu.ft. of air				
						During cleaning		After cleaning	
		Before cleaning (initial count)	After cleaning	Mean % of initial count	Before cleaning (initial count)	No.	Mean % of initial count	No.	Mean % of initial count
Broom	4	4·19	5·16	140·7	0·37	1·28	346·9	1·00	268·7
Fresh oiled mop	4	7·10	4·39	70·7	0·50	0·80	203·3	0·783	145·1
Oiled mop used 7 times	3	4·54	2·13	73·8	0·29	0·58	300·0	0·44	233·3
Vacuum cleaner	4	5·78	3·25	67·7	0·35	0·24	116·7	0·41	112·5

Impressions of use

The mean times taken in cleaning the ward by the three methods were as follows: oiled mops (two in use, one of 20 in. and one of 12 in.): 12·3 min. (S.E. = 1·0), vacuum cleaner: 37·8 min. (S.E. 3·0), brooms (two in use): 12·6 min. (S.E. = 1·4).

The broom caused an unpleasant cloud of dust in both wards. Airborne dust was not noticed when using the oiled mops or the vacuum cleaner. The floor surface of ward I, which was unoiled, acquired a pleasant slightly polished appearance after repeated use of the oiled mop.

COMMENTS

In their removal of bacteria from the floor oiled mops compare favourably with the vacuum cleaner. The fresh oiled mop is also effective in reducing the dispersal of floor dust into the air, but although the results are sometimes as good as those obtained with a vacuum cleaner, they vary considerably. Oiled mops have certain advantages; they are quicker, quieter and easier to use than vacuum cleaners; in addition they give the floor a clean and lightly polished appearance. On repeated use they disperse large numbers of bacteria into the air; although these settle more quickly than the bacterial dust raised by a broom, it is probably desirable to cleanse and re-impregnate mops after a single use.

SUMMARY

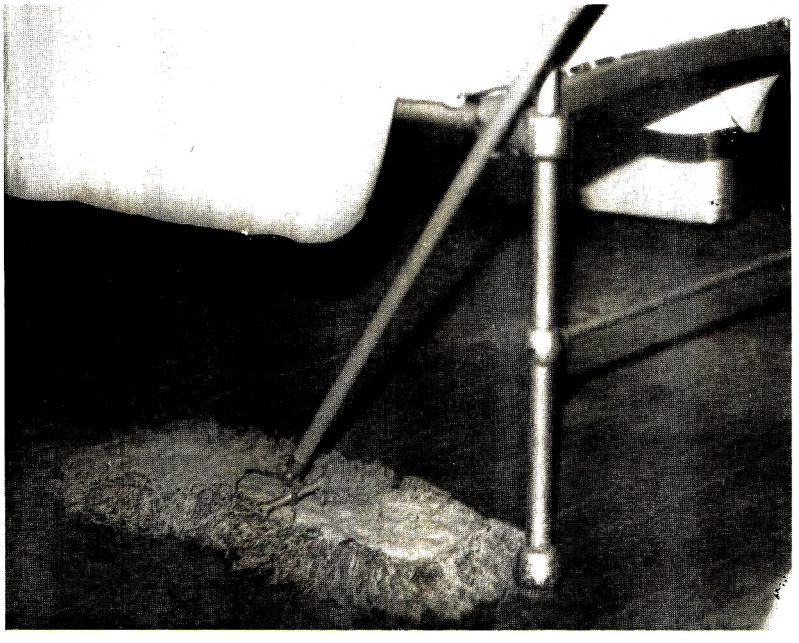
A comparison was made of three methods for the removal of dust from a hospital ward floor.

The viable bacterial counts of impression plates from the floor showed a mean reduction of 51 % after cleaning with fresh oiled mops, 40 % after cleaning with a tank model vacuum cleaner and 5 % after sweeping with a broom.

The mean proportions of airborne bacteria, compared with viable counts before cleaning, were 192 % during and 194 % after cleaning with a broom; 128 % during and 103 % after cleaning with an oiled mop, and 82 % during and 48 % after cleaning with a vacuum cleaner. On repeated use the oiled mop dispersed almost as much dust as a broom, but this settled rapidly from the air.

The effects of these cleaning methods on counts of presumptive *Staph. aureus* on the floor and in the air were similar to those found in counts of total organisms.

We wish to thank Mr M. D. Wilkins for valuable assistance, the Domestic Superintendent and staff and the nursing staff for their co-operation, and Messrs Leeming Brothers Limited for supplies of 'Kex' mops and equipment.



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EXPLANATION OF PLATE

An oiled mop (20 in.) in use on a ward floor.

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Optimum moving averages for the estimation of median effective dose in bioassay

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1. INTRODUCTION AND STATEMENT OF PROBLEMS

Suppose that $x_1, x_2, \ldots, x_t$ represent the numbers of subjects in which an all-or-none response (e.g. death) is observed to occur amongst the $n_1, n_2, \ldots, n_t$ subjects independently tested at t different dose levels $d_1, d_2, \ldots, d_t$ of a certain substance (e.g. drug or hormone). The problems of quantal response in bioassay may be formulated as follows. If $F(d)$ represents an assumed distribution, or the proportion π expected to react at a dose d or less, then the x's each have independent binomical distributions

$$\frac{n_i!}{x_i!(n_i-x_i)!}\pi_i^{x_i}(1-\pi_i)^{n_i-x_i} \tag{1}$$

for $i = 1, 2, \ldots, t$ and $0 \leqslant x_i \leqslant n_i$.

Principal interest has been shown thus far in estimation of the parameters and the dose-response relation from the assumed distribution

$$F(d) = \frac{1}{\sqrt{2\pi}\sigma}\int_{-\infty}^{d} \exp\left[-\frac{1}{2\sigma^2}(u-\mu)^2\right]du, \tag{2}$$

i.e. the cumulative normal distribution (e.g. Finney, 1952), and also from the 'logistic' distribution

$$F(d) = [1+\exp[-(\alpha+\beta d)]^{-1}] \tag{3}$$

(e.g. Berkson, 1953).

When nothing is assumed about the nature of the probabilities in the form of a specified response distribution $F(d)$ as above, Thompson (1947) proposed the use of 'moving averages' as a method of estimating the median effective or lethal dose (= LD 50). An (unweighted) 'moving average' of span k is defined as the successive sums

$$p_i' = \frac{1}{k}\sum_{r=0}^{k-1}\left(\frac{x_{i+r}}{n_{i+r}}\right) = \frac{1}{k}\sum_{r=0}^{k-1} p_{i+r} \tag{4}$$

in terms of the sample proportions p, and an associated dose

$$d_i' = \frac{1}{k}\left(\sum_{r=0}^{k-1} d_{i+r}\right). \tag{5}$$

In order to estimate LD 50 Thompson used ordinary linear interpolation between consecutive values of the moving average p' on either side of 0·50. Finney (1952, ch. 20) has discussed the efficiency of these unweighted moving averages with reference to the integrated normal distribution (2).

The present author (1952) has made one modification in moving averages by introducing weights proportional to the n's for odd-numbered spans of 3, 5, etc. Thus, for example, for three-term moving averages

$$p_i' = \left(p_{i-1} + \frac{n_i}{\bar{n}_i} p_i + p_{i+1}\right) \Big/ \left(2 + \frac{n_i}{\bar{n}_i}\right) \tag{6}$$

and a corresponding dose d_i' in terms of the harmonic mean $\bar{n}_i$ defined by

$$\frac{1}{\bar{n}_i} = \frac{1}{2}\left(\frac{1}{n_{i-1}} + \frac{1}{n_{i+1}}\right),$$

and the author has also discussed the efficiency of this form of moving averages in estimating LD 50. If the n's all happen to be equal, then the average (6) above coincides with the averages defined by Thompson.

It is the purpose of this paper to present some further theoretical aspects of determining new optimum forms of moving averages in estimation of LD 50 together with their approximate sampling errors.

2. GENERALITIES ON MOVING AVERAGES

For the $(2k+1)$ sample proportions $\{p_{i+r}\}(r = 0, \pm 1, \ldots, \pm k)$, consider the minimization of the weighted sums of squares

$$S_i = \sum_{r=-k}^{+k} \lambda_{i+r}(p_{i+r} - \pi_i)^2 \tag{7}$$

with respect to π_i for each $i = k+1, \ldots, (t-k)$, and subject to the further condition

$$\sum_{r=-k}^{+k} \lambda_{i+r} = 1.$$

This minimization results in estimates of the form $p_i' = \hat{\pi}_i = \sum_{r=-k}^{+k} \lambda_{i+r} p_{i+r}$, which will be called a moving average of span $(2k+1)$.

It should be mentioned that the condition $\sum_r \lambda_{i+r} = 1$ results from the natural requirement that if the observed proportions happen to coincide, i.e. if $p_{i+r} = p_i^0$, $r = 0, \pm 1, \ldots, \pm k$, then $p_i' = p_i^0$ as a reasonable condition of the moving average.

If then the variance of each p_i'

$$V(p_i') = \sum_{r=-k}^{+k} \lambda_{i+r}^2 \frac{\pi_{i+r}(1-\pi_{i+r})}{n_{i+r}} \tag{8}$$

is minimized for variation in the λ's, it is easy to verify that this occurs when

$$\hat{\lambda}_{i+r} = \frac{n_{i+r}\pi_i}{\pi_{i+r}(1-\pi_{i-r})} \Big/ \sum_{r=-k}^{+k} \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}, \tag{9}$$

i.e. the weights are proportional to the reciprocals of the (binomial) weights for each sample proportion. The resulting minimum variance is

$$V(p_i') = \left[\sum_{r=-k}^{+k} \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}\right]^{-1}.$$

If the true proportions π are known, then LD 50 is to be estimated by linear interpolation between the two consecutive p''s on either side of 0·5 in terms of the transformed doses

$$d'_i = \sum_{r=-k}^{+k} \hat{\lambda}_{i+r} d_{i+r}.$$

In particular, if the original doses are equally spaced, i.e. if $d_i = d_0 + (i-1)d$ in terms of an initial (log) dose level ($= d_0$) and uniform spacing d, the resulting spacing for the d''s is

$$d'_i = d_0 + \left\{(i-1) + \left(\sum_{r=-k}^{+k} r\lambda_{i+r}\right)\right\} d \tag{10}$$

which will be in fact equal only in case of a completely symmetric set of the weights, i.e. $\lambda_{i-r} = \lambda_{i+r}$, for $r = 1, 2, \ldots, k$. This can occur when equal numbers are used with a symmetric dose-response or in terms of the population proportions, $\pi_{i-r} = \pi_{i+r}$.

3. UNBIASED MOVING AVERAGES

If now it is required only that each successive p'_i be an unbiased estimate of the corresponding π_i, i.e.

$$E(p'_i) = \sum_{r=-k}^{+k} \lambda_{i+r}\pi_{i+r} = \pi_i,$$

the weights resulting from minimizing the variance (8) subject to the condition of unbiasedness are

$$\begin{aligned} \hat{\lambda}_{i+r} &= \frac{n_{i+r}\pi_i}{(1-\pi_{i+r})} \Big/ \left[\sum_{r=-k}^{+k} n_{i+r}\left(\frac{\pi_{i+r}}{1-\pi_{i+r}}\right)\right] \\ &= \left(\frac{\pi_i}{\pi_{i+r}}\right) n_{i+r}\, e^{Y_{i+r}} \Big/ \left[\sum_{r=-k}^{k} n_{i+r}\left(\frac{\pi_{i+r}}{1-\pi_{i+r}}\right)\right] \end{aligned} \tag{11}$$

in terms of the usual 'logit' transformation $Y_i = \log_e[\pi_i(1-\pi_i)^{-1}]$. The minimum variance is then

$$V(p'_i) = (\pi_i^2)\left[\sum_{r=-k}^{k} n_{i+r}\left(\frac{\pi_{i+r}}{1-\pi_{i+r}}\right)\right]^{-1} = (\pi_i^2)\left[\sum_{-k}^{k} n_{i+r}\, e^{Y_{i+r}}\right]^{-1}.$$

If the requirements on the λ's of being both unbiased and such that

$$\sum_r \lambda_{i+r} = 1$$

are superimposed, then minimum variance is attained whenever

$$\hat{\lambda}_{i+r} = \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}\left(\frac{p_{ir}}{\Delta_i}\right), \tag{12}$$

where

$$\begin{aligned} p_{ir} &= \left[\sum_{j=-k}^{+k} \frac{n_{i+j}(\pi_{i+j}-\pi_i)(\pi_{i+j}-\pi_{i+r})}{\pi_{i+j}(1-\pi_{i+j})}\right], \\ \Delta_i &= \left[\sum_r \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}\right]\left[\sum_r \frac{n_{i+r}\pi_{i+r}}{(1-\pi_{i+r})}\right] \\ &\quad - \left[\sum_r \frac{n_{i+r}}{(1-\pi_{i+r})}\right]^2. \end{aligned} \tag{13}$$

It is seen that the weights or coefficients $\hat{\lambda}$ obtained in equations (11) and (12) are parametric multiples of the binomial weights in (9).

In view then of the sampling complications of weights based on equations (11) or (12), it is felt that successive moving averages based on sample estimates of the binomial weights (9) are to be considered adequate in estimation of LD 50. These estimates together with their approximate standard errors will now be derived.

4. SAMPLING THEORY WHEN BINOMIAL WEIGHTS ARE USED

In applying the results of § 3 for the binomial weights (9), we replace the π's by their corresponding sample estimates p. The moving averages p' are then computed from the sample weights

$$\hat{\lambda}_{i+r} = \frac{n_{i+r}}{p_{i+r}(1-p_{i+r})} \Big/ \left[\sum_{r=-k}^{k} \frac{n_{i+r}}{p_{i+r}(1-p_{i+r})}\right] \tag{14}$$

excluding any observed zero or one values for the sample p's. In these latter cases the value of the observed numbers x should be replaced by 1 or $(n-1)$, respectively in the calculations.

If then two successive moving average proportions p'_i, p'_{i+1} are such that $p'_i < 0{\cdot}5 < p'_{i+1}$, the estimate of LD 50 obtained by linear interpolation is

$$m' = d'_i + \frac{(0{\cdot}5 - p'_i)}{(p'_{i+1} - p'_i)}(d'_{i+1} - d'_i) \tag{15}$$

with variance

$$V(m') = (d'_{i+1} - d'_i)^2 V\left(\frac{0{\cdot}5 - p'_i}{p'_{i+1} - p'_i}\right). \tag{16}$$

Using the formula for the approximate variance of a ratio of two random variables, we obtain

$$V(m) \cong \frac{(d'_{i+1} - d'_i)^2}{(\pi'_{i+1} - \pi'_i)^2}[(1-\tau)^2 V(p'_i) + 2\tau(1-\tau)C(p'_i, p'_{i+1}) + \tau^2 V(p'_{i+1})], \tag{17}$$

where π'_i are the corresponding moving averages of the true proportions π again estimated from the sample and

$$V(p'_i) = \left[\sum_{r=-k}^{+k} \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}\right]^{-1}, \tag{18}$$

$$C(p'_i, p'_{i+1}) = \left[\sum_{r=-(k-1)}^{k} \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}\right] \Big/ \left[\sum_{r=-k}^{k} \frac{n_{i+r}}{\pi_{i+r}(1-\pi_{i+r})}\right] \left[\sum_{-k}^{k} \frac{n_{i+r+1}}{\pi_{i+r+1}(1-\pi_{i+r+1})}\right]$$

if we denote the fraction $\tau = (0{\cdot}5 - p'_i)/(p'_{i+1} - p'_i)$.

5. OPTIMUM MOVING AVERAGES BASED ON ANGULAR TRANSFORMATION

Finally, it is of interest to point out an optimum form of moving average based on the arc-sine transformation of proportions (e.g. Snedecor, 1956, p. 318), which is well known for the property of approximately stabilizing the variance. In terms of the sample proportions $p_i = x_i/n_i (i = 1, \ldots, c)$, the arc-sine transformation

$$y_i = \sin^{-1}\sqrt{p_i}$$

is such that the resulting y's have approximately constant variance $V(y_i) = c^2/n_i$, where

$$c^2 = 0{\cdot}25 \quad \text{if } y \text{ in radians}$$
$$= 821 \quad \text{if } y \text{ in degrees.}$$

Moving averages on the y's of the form $y'_i = \sum_{r=-k}^{+k} \lambda_{i+r} y_{i+r}$ attain minimum variance when

$$\hat{\lambda}_{i+r} = n_{i+r} \Big/ \left[\sum_{r=-k}^{k} n_{i+r}\right],$$

and in this case

$$V(y'_i) = c^2 \Big/ \left[\sum_{r=-k}^{k} n_{i+r}\right]. \tag{19}$$

The corresponding doses are

$$d_i^* = \left(\sum_{r=-k}^{k} n_{i+r} d_{i+r}\right) \Big/ \left[\sum_{r=-k}^{k} n_{i+r}\right] \tag{20}$$

which, in case the d's are equally spaced $d_i = d_0 + (i-1)\,d$ with interval d, reduce to

$$d_i^* = d_0 + \left\{(i-1) + \frac{(\Sigma r n_{i+r})}{(\Sigma n_{i+r})}\right\} d. \tag{21}$$

If now two successive values y'_i, y'_{i+1} of the sequence of moving averages are such that $y'_i < \frac{1}{4}\pi < y'_{i+1}$, then the estimate m^* of LD 50 will be

$$m^* = d_i^* + \frac{(\frac{1}{4}\pi - y'_i)}{(y'_{i+1} - y'_i)} (d_{i+1}^* - d_i^*) \tag{22}$$

with approximate variance

$$V(m^*) = \frac{(d_{i+1}^* - d_i^*)^2}{(\theta'_{i+1} - \theta'_i)^2} [(1-\nu)^2 V(y'_i) + 2\nu(1-\nu) C(y'_i, y'_{i+1}) + \nu^2 V(y'_{i+1})], \tag{23}$$

where

$$\theta_i = \sin^{-1}\sqrt{\pi_i},$$

$$V(y'_i) = (c)^2 \Big/ \left(\sum_{r=-k}^{k} n_{i+r}\right), \tag{24}$$

$$C(y'_i, y'_{i+1}) = (c^2) \left[\sum_{r=-(k-1)}^{k} n_{i+r}\right] \Big/ \left[\sum_{r=-k}^{k} n_{i+r}\right] \left[\sum_{r=-k}^{k} n_{i+r+1}\right]$$

and θ'_i is the weighted average $= (\Sigma n_{i+r}\theta_{i+r})/(\Sigma n_{i+r})$, and ν is the fraction $\nu = (\frac{1}{4}\pi - \theta'_i)/(\theta'_{i+1} - \theta'_i)$.

Table 1. *Toxicity of rotenone* (*Finney*, 1947)

(D) concentration mg./l.	$\log\left(\frac{D}{D_0}\right) = d_i$	No. of insects n_i	No. affected x_i	$100\,p_i$	Degrees y_i	y'_i	d_i^*
$2{\cdot}6 = D_0$	0·0000	50	6	12·0	20·27	—	—
3·8	0·1644	48	16	33·3	35·24	33·56	0·1482
5·1	0·2923	46	24	52·2	46·26	49·35	0·3107
7·7	0·4713	49	42	85·7	67·78	61·63	0·4566
10·2	0·5933	50	44	88·0	69·73	—	—

6. EXAMPLE

The following example of an optimum three-term moving average using the arc-sine transformation is based on the data (Table 1) on toxicity of rotenone when sprayed on *Macrosiphoniella sanborni* in batches of approximately fifty insects each (Finney, 1947, p. 26).

Using equations (22) and (23), we obtain the estimates

$$\begin{aligned} m^* &= \log_{10}(\text{LD } 50) = \log 2{\cdot}6 + 0{\cdot}1482 + \frac{(45 - 33{\cdot}56)}{(49{\cdot}35 - 33{\cdot}56)}(0{\cdot}1625) \\ &= 0{\cdot}4150 + 0{\cdot}1482 + (0{\cdot}7245)(0{\cdot}1625) = 0{\cdot}6809, \\ V(m^*) &= \frac{(0{\cdot}1625)^2}{(15{\cdot}79)^2}[(1 - 0{\cdot}7245)^2 V(y_i') + 2(0{\cdot}7245)(0{\cdot}2755)\, C(y_i', y_{i+1}) \\ &\quad + (0{\cdot}7245)^2 V(y_{i+1})] \\ &= \frac{(0{\cdot}02641)}{249{\cdot}32}(4{\cdot}9424) = 5{\cdot}235 \times 10^{-4}. \end{aligned}$$

Table 2 compares this estimate and its variance with the corresponding maximum likelihood estimate (Finney, 1947) and the minimum logit χ^2 value (Berkson, 1953) with their respective variances.

Table 2. *Comparison of estimates of log (LD 50)*

Method	Estimate	Variance ($\times 10^4$)
Maximum likelihood (probits)	0·6862	4·849
Minimum (logit) χ^2	0·6848	5·215
Optimum moving average (arc-sine)	0·6809	5·235

Table 2 demonstrates the relatively high efficiency of a three-term moving average based on the angular transformation when compared with an assumed cumulative normal (92·6 %) and the logit (99·6 %) in this example.

SUMMARY

Optimum forms of moving averages are derived for the estimation of LD 50 in the situation where no assumptions are made about the form of the dose-response distribution. The theory is also applied to uses of moving averages based on the angular transformation of the percentage response. A numerical example illustrates this application, and its results are compared with the corresponding probit and logit estimates.

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Para-influenza 2 virus infections in adult volunteers

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INTRODUCTION

Para-influenza viruses 1, 2 and 3 produce illnesses in children which range from mild upper respiratory infections to severe diseases of the lower respiratory tract (Parrott, Vargosko, Kim, Bell & Chanock, 1962). Para-influenza 2 virus is isolated from children much less frequently than para-influenza 1 or 3 viruses and does not often seem to be associated with illness other than croup (Kim, Vargosko, Chanock & Parrott, 1961). Para-influenza viruses have also been isolated from adults with mild upper respiratory infections (Evans, 1960; Holland, Tanner, Pereira & Taylor, 1960; Dick, Mogabgab & Holmes, 1961; Mogabgab, Dick & Holmes, 1961), but it has been difficult to prove that these viruses are aetiologically associated with the illnesses that have been observed. This has been due to the fact that the para-influenza viruses have been isolated along with many other different viruses and have formed only a very small portion of the total number of viruses isolated. However, para-influenza viruses 1 and 3 are able to produce upper respiratory infections of a mild cold-like nature in adult volunteers (Reichelderfer, Chanock, Craighead, Huebner, Turner, James & Ward, 1958; Tyrrell, Bynoe, Birkum Petersen, Sutton & Pereira, 1959; Kapikian, Chanock, Reichelderfer, Ward, Huebner & Bell, 1961). We present evidence that para-influenza 2 virus also produces mild upper respiratory illness in adult volunteers inoculated intranasally.

MATERIALS AND METHODS

Viruses

The para-influenza 2 virus used for volunteer inoculation was obtained from Dr M. S. Pereira (Central Public Health Laboratory, Colindale, London) as freeze-dried tissue culture fluid from a 4th monkey kidney passage. This material was reconstituted and inoculated into primary trypsin-dispersed cultures of human embryo kidney cells and these cultures were rolled and incubated at 33° C. The tissue-culture fluids were pooled on the 4th day of incubation, bovine plasma albumin was added to a final concentration of 1·0% and the pooled fluids were divided into 2·0 ml. aliquots and stored at −70° C. until used. This virus was passed once more in rhesus monkey kidney cells and was stored at −70° C. until used in neutralization tests.

The following strains propagated in tissue culture were used in haemaggluti-

nation-inhibition tests: para-influenza 2—Manganero strain; para-influenza 1—Copenhagen 222 strain; para-influenza 3—bovine strain 33; and para-influenza 3—human strain C243.

Safety tests

The virus for volunteer inoculation was neutralized by a para-influenza 2 rabbit antiserum, but not by antisera to para-influenza viruses 1 and 3. The virus ($10^{7.45}$ tissue-culture doses (TCD 50) per ml.) was inoculated intravenously (1·0 ml.) and intramuscularly (1·0 ml.) into a rabbit, intramuscularly (0·5 ml.) into guinea-pigs and intracerebrally (0·05 ml.) and intraperitoneally (0·1 ml.) into adult mice and intracerebrally (0·03 ml.) and intraperitoneally (0·03 ml.) into unweaned mice; no illnesses were observed. Tests for the presence of simian virus 40 in the para-influenza 2 virus pool were made by inoculating mixtures of virus and serum from a rabbit immunized against another strain of para-influenza 2 virus into vervet monkey kidney cells; the cultures were observed for 2 weeks and subcultures from these were also observed for 2 weeks. These tests were carried out by Dr D. McGrath (Medical Research Council Laboratories, Holly Hill, Hampstead); simian virus 40 was not detected.

Volunteers

The volunteers were between 18 and 50 years of age. They were isolated as described elsewhere (Andrewes, 1948), usually in pairs, but occasionally singly or in groups of three. After an observation period of 3½ days virus was given as nasal drops in a volume of 1·0 ml. of chilled Hanks's saline. Blood was collected 1 or 2 days before virus inoculation and again 14–16 days after inoculation when the volunteers had left the unit. Nasal washings were collected as described below. Each volunteer was examined daily by one of us (M. L. B.) who was unaware of the nature of the inoculum which each had received.

Tissue cultures

Primary trypsin-dispersed cultures of human embryo kidney cells and secondary cultures of rhesus monkey kidney cells were grown in a medium of 5 % calf serum, 0·5 % lactalbumin hydrolysate and Hanks's saline containing 0·03 % sodium bicarbonate and antibiotics. Before use, the cultures were washed three times with Hanks's saline and then maintained in medium 199. Primary cultures of calf kidney were grown in a medium which contained 10 % calf serum, 0·5 % lactalbumin hydrolysate and Hanks's saline containing 0·03 % sodium bicarbonate. Again, the cultures were washed three times with Hanks's saline and then maintained in 0·5 % lactalbumin hydrolysate, 0·1 % yeast extract and Hanks's saline containing 0·1 % sodium bicarbonate. HeLa cells, obtained from Dr M. S. Pereira, were grown in medium consisting of 10 % rabbit serum, 0·5 % lactalbumin hydrolysate, 0·1 % yeast extract and Hanks's saline containing 0·1 % sodium bicarbonate and antibiotics. These cultures were also washed and maintained in medium 199.

Virus isolation

Nasal washings in Hanks's saline were usually collected 2, 3, 4 and 5 days after intranasal inoculation of virus. The washing from each volunteer was chilled in crushed ice and, within an hour of being taken, 0·2 ml. was inoculated into each of two cultures of human embryo kidney cells. The cultures were rolled at 33° C. Some washings were titrated by inoculating 10-fold dilutions made in Hanks's saline. Cultures were examined for the typical syncytial cytopathic effect of para-influenza 2 virus, but the final recording was based on the haemadsorption test (Vogel & Shelokov, 1957); 4–5 days after inoculation, 0·2 ml. of a 1·0% suspension of human group 'O' red cells in phosphate-buffered saline was added to each tube and the tubes were placed at 4° C. for 30 min. and were then read. Virus detected in this way was then identified by haemadsorption-inhibition with specific rabbit antiserum (Chanock, Parrott, Cook, Andrews, Bell, Reichelderfer, Kapikian, Mastrota & Huebner, 1958). Fresh medium was added to those cultures that did not exhibit haemadsorption and they were incubated for a further 6–7 days and retested.

Neutralization tests

Serial twofold dilutions of serum were mixed with an equal volume of a dilution of virus containing about 100 TCD 50 in 0·1 ml. After 30 min. incubation at room temperature, 0·2 ml. of each mixture was inoculated into each of two rhesus monkey kidney cultures which were then rolled at 33° C. Five days later, the cultures were tested by haemadsorption. Neutralization titres were expressed as interpolated 50% end-points. Chanock (1956) found that the neutralizing activity of sera from children was lost on heating, but partly restored on adding unheated normal serum. We did not experience this difficulty. There was no significant difference between titres obtained with sera inactivated at 56° C. for 30 min. and sera not so treated and, therefore, we used inactivated sera. Titres in all the serological tests are expressed as the reciprocal of the initial serum dilution.

Complement-fixation tests

Some para-influenza 2 complement-fixing antigen was obtained from Dr P. Bradstreet (Central Public Health Laboratory, Colindale, London) and some was prepared by ourselves in HeLa cells; the culture fluids were used as antigen. All sera were inactivated at 56° C. for 30 min. before use. The tests were done in WHO haemagglutination plates with one drop of each reagent. Two units of complement and overnight fixation at 4° C. were used.

Haemagglutination-inhibition (H.I.) *tests*

All the acute and convalescent sera were treated with cholera filtrate, and tested against para-influenza viruses 1, 2 and 3. Para-influenza 1 haemagglutinin was prepared in human embryo kidney cells; the culture fluids were used as antigen. Para-influenza 2 antigen was prepared in human embryo kidney and in HeLa cells; the cells were disrupted by blending and the fluids from both types of culture were then pooled, centrifuged at 2000 r.p.m. for 10 min. and the

supernatant used as the antigen. Haemagglutinins were prepared from the human and bovine strains of para-influenza 3 in human embryo kidney cells and in calf kidney cells, respectively; the tissue culture fluids were used as antigens. Preliminary experiments showed that the same H.I. titres were obtained when sera from adult humans were tested with either the bovine strain or the human strain. Thereafter, only the bovine strain was used because it was easier to produce haemagglutinin from it than from the human strain. H.I. tests with para-influenza viruses 1 and 3 were done in WHO plates with 0·2 ml. volumes. Four units of antigen were incubated with dilutions of serum at room temperature for 60 min.; then 1·0 % human group 'O' red cells were added and allowed to settle at 4° C. In order to save virus, tests with para-influenza 2 haemagglutinin were carried out by the micro-method of Takátsy (Takátsy, 1955) and 1·0 % chicken erythrocytes were used.

RESULTS

Clinical evidence of infection

Twenty-eight volunteers were inoculated intranasally with para-influenza 2 virus and twenty-eight were given Hanks's saline alone. From 2×10^2 to 2×10^6 TCD 50 of virus were given to each volunteer and the clinical findings are shown in Table 1. Most of the symptoms and signs were of moderate severity; and nasal stuffiness and sore throat were more evident than coryza. Eight volunteers showed enough symptoms and signs to convince the observer that they had a significant illness, but only four of these illnesses were typical experimental colds (Roden, 1958). The incubation period was 2 days for three of the colds and 4 days for the other. The other four illnesses had incubation periods of 2, 2, 3 and 3 days, respectively. Of the twenty-eight volunteers inoculated with virus, eighteen had at least one symptom or sign and there was a total of fifty-one symptoms or signs. Of twenty-eight volunteers given Hanks's saline, six had symptoms or signs of which there was a total of twelve; most of these symptoms and signs were due to one subject who had a mild cold. This evidence suggests that most of the symptoms of those subjects given the virus-containing inoculum were due to the virus it contained. Only one of six volunteers who were inoculated with 2×10^4 TCD 50 of virus became ill and no volunteer given less virus than this was ill. Three illnesses occurred in eleven volunteers inoculated with 2×10^5 TCD 50 of virus and four illnesses in six subjects inoculated with 2×10^6 TCD 50; this suggests that large doses of virus produced more illness than small ones. It is possible that the colds reported by volunteers nos. 8, 9 and 28 were due to the experimental virus infection. However, since these colds developed 7, 6 and 6 days respectively after inoculation and after the period of strict isolation they have not been included in the results.

Laboratory evidence of infection

Laboratory evidence of infection was based on the re-isolation of virus or a fourfold antibody response as determined by any of the three serological tests used.

Table 1. *The symptoms and signs, clinical assessment, virus isolations and antibody responses of twenty-eight volunteers inoculated intranasally with para-influenza 2 virus*

Volunteers (no.)	Virus dose (TCD 50) for each volunteer	Symptoms and signs: Headache	Chills	Pyrexia	Sneezing	Coryza	Max. no. of paper handkerchiefs per day	Nasal stuffiness	Sore throat	Injected fauces	Clinical assessment	Virus isolations from nasal washings. Days after inoculation of volunteer: 2	3	4	5	Reciprocal antibody titres of pre- and post-inoculation sera as shown by: Neutralization		Haemagglutination inhibition		Complement fixation	
1	2×10^2	.	.	.	.	.	.	.	.	.	.					N.T.‡		< 8	8	< 4	< 4
2		.	.	.	.	.	.	.	.	.	.					N.T.		8	12	< 8	8
3	2×10^3	.	.	.	.	.	.	.	.	+	.	N.T.			N.T.	48	64	12	12	32	32
4		.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	< 8	< 8	< 8	8	4	4
5		.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	**12**	**48**	8	12	4	8
6	2×10^4	.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	**8**	**48**	< 8	**32**	**4**	**16**
7		.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	24	48	< 8	< 8	4	4
8		+	.	.	+	+	4	.	.	+	U.R.I.*					**8**	**128**	< 8	**48**	< **4**	**12**
9		+	.	.	.	.	.	.	.	.	.					**24**	**96**	**8**	**32**	**16**	**64**
10		.	.	.	.	.	.	+	.	.	.					**8**	**64**	**8**	**32**	**4**	**32**
11		.	.	.	.	.	.	.	+	.	.					16	24	12	24	**16**	**64**
12	2×10^5	+	+	.	.	+	6	+	+	+	Cold (2)†					**16**	**96**	**8**	**32**	**4**	**32**
13		+	+	.	+	+	12	+	+	+	Cold (2)					**24**	**96**	**8**	**32**	< **8**	**32**
14		.	.	.	.	.	.	.	.	.	.					48	48	24	24	16	16
15		.	.	99·2	.	.	.	.	.	.	.					**8**	**32**	8	16	**4**	**16**
16		.	.	.	.	.	.	.	.	.	.					12	12	8	8	8	8
17		.	.	.	.	.	.	.	.	.	.					48	48	12	8	< 8	8
18		.	+	99·2	+	+	12	+	+	.	Cold (2)					< **8**	**64**	**8**	**48**	**4**	> **64**
19		.	.	.	.	.	.	+	.	.	.					< **8**	**48**	< **8**	**32**	**4**	> **64**
20		.	.	.	.	.	.	+	.	.	.					< **8**	**32**	< **8**	**24**		
21		.	.	.	+	.	.	+	+	.	.					< 8	< 16	8	16	16	16
22		.	.	.	.	.	.	.	.	.	.				N.T.	< **8**	**32**	**12**	**48**	< **4**	**32**
23	2×10^6	.	.	99·4	+	+	10	+	.	.	Cold (4)					64	96	24	32	**8**	**32**
24		.	.	.	.	.	.	+	.	.	.					16	24	12	24	**4**	**16**
25		+	.	.	+	.	.	.	+	.	U.R.I.					24	16	< 8	< 8	4	4
26		+	.	.	.	.	.	+	+	+	U.R.I.					12	24	8	12	8	8
27		+	+	.	+	.	.	+	+	.	U.R.I.					24	32	8	12	8	8
28		.	.	.	+	.	.	.	.	.	.					**12**	**128**	**8**	**48**	**8**	**32**

Figures in bold type in the last three columns indicate a fourfold or greater rise of antibody titre.
* U.R.I. = upper respiratory infection. † In parentheses, the incubation period. ‡ N.T. = not tested. □ = virus not isolated. ▨ = virus isolated.

Virus isolations

Although there was no evidence that human embryo kidney cells were more sensitive than rhesus monkey kidney cells, we used the former in order to avoid haemadsorption due to simian haemadsorbing agents. As shown in Table 1, parainfluenza 2 virus was re-isolated from all but one of the volunteers who were given 2×10^4 or more TCD 50 of virus. This correlates with the clinical picture since no volunteer given less than 2×10^4 TCD 50 of virus was considered to have clinical evidence of disease. However, fourteen volunteers were infected, as judged by virus isolation, although they did not have clinical evidence of infection. Virus was re-isolated from nine of twenty nasal washings taken from volunteers inoculated with 2×10^4 TCD 50 of virus and from twenty-four of twenty-four washings taken from volunteers inoculated with 2×10^6 TCD 50 of virus. This is a significant difference at the 1% level and suggests that virus was excreted less frequently from those given small amounts of virus.

Table 2. *The amount of virus in nasal washings of volunteers inoculated intranasally with* 2×10^6 TCD 50 *of virus*

	Virus titre (TCD 50/0·6 ml.) in washings collected at various times after original virus inoculation				
Volunteer	9 hr.	2 days	3 days	4 days	5 days
25	0	$10^{1.5}$	$10^{1.5}$	$10^{1.5}$	$10^{0.7}$
26	0	$10^{2.0}$	$10^{2.0}$	$10^{2.0}$	$10^{1.0}$
27	0	$10^{0.5}$	$10^{0.7}$	$10^{0.5}$	10^{0}
28	0	$10^{2.0}$	$10^{2.0}$	$10^{2.0}$	$10^{2.0}$

The virus that was re-isolated from volunteers was unlikely to be residual virus from the inoculum as shown in the following experiment (Table 2). Four volunteers were each inoculated with 2×10^6 TCD 50 of virus; virus could not be re-isolated from nasal washings taken 9 hr. later, but virus was isolated 2, 3, 4 and 5 days after inoculation. Further, virus was isolated on the 5th day after inoculation from sixteen volunteers (Table 1) and this virus must almost certainly have been produced in the respiratory tract and not have been residual virus from the inoculum.

It may be seen from Table 2 that volunteer no. 28, who was not ill, consistently excreted 10-fold more virus than volunteer no. 27, who had an upper respiratory illness. It appears, therefore, that the amount of virus re-isolated was unrelated to the presence of overt illness.

Antibody responses

As shown in Table 1, the four persons who had cold-like illnesses and five of the eight volunteers who were considered to have clinical evidence of infection had a fourfold antibody response in at least one of the serological tests. There was no evidence that volunteers who were given large doses of virus developed higher titres of antibody than those given the smaller doses; in fact, three of the six persons who were given 2×10^6 TCD 50 of virus had no antibody response at all.

There was a greater chance of detecting a fourfold neutralizing antibody rise in infected volunteers who had initial low levels of antibody than in those with initial high levels; ten of thirteen volunteers with neutralizing antibody titres of twelve or less showed an antibody response, whereas only three of eleven volunteers with antibody titres of sixteen or more showed a response. Antibody rises were detected in thirteen instances by neutralization tests, in eleven cases by H.I. tests and in fourteen cases by complement-fixation tests. However, although an antibody response was detected by all three tests in ten instances, it was shown by only one technique in four instances and it was necessary, therefore, to titrate antibodies by the three serological techniques in order to detect all the responses. An antibody response was a less sensitive indicator of infection than virus isolation, since virus was isolated from eight individuals who did not show an antibody response by any of the three serological techniques. In only two instances was there an antibody response without virus isolation.

As shown in Table 1, twenty volunteers had neutralizing antibody titres of eight or more before inoculation of virus so it seems likely that these subjects had been infected previously, probably in childhood, and that the illnesses observed were due to re-infection with para-influenza 2 virus in the presence of antibody. Too few volunteers were studied to establish whether a high level of neutralizing antibody could protect completely or whether a low level protected partially. It was noted, however, that low levels of antibody certainly failed to protect completely against re-infection, for persons with titres of 24 were infected by 2×10^4 TCD 50 of virus and one subject with a titre of 64 was not protected against 2×10^6 TCD 50 of virus.

Table 3. *Result of para-influenza 2 virus neutralization test carried out* in vivo

	No. of volunteers				
Intranasal inoculum	Inoculated	From whom virus isolated	With fourfold antibody rise	With symptoms and signs	With colds
2×10^5 TCD 50 of virus	5	5	4	4	1
2×10^5 TCD 50 of virus-antiserum mixture	6	0	0	1	0

When the paired sera were tested by haemagglutination-inhibition against Sendai virus and para-influenza viruses 1 and 3 no antibody rises were detected. The same paired sera were tested by Dr R. B. Heath (St Bartholomew's Hospital Medical School, London) against mumps virus by the H.I. test and again no rises were detected.

A neutralization test carried out in vivo

Because volunteers became ill even in the presence of neutralizing antibody it was possible that these illnesses were due not to para-influenza 2 virus, but to an undetected virus in the inoculum. The following experiment was, therefore, carried

out. The para-influenza 2 virus pool was diluted to contain approximately 2×10^5 TCD 50 of virus per ml. and this was mixed with an equal volume of para-influenza 2 rabbit antiserum. Virus, similarly diluted, was mixed with an equal volume of Hanks's saline. The mixtures were incubated at room temperature for 30 min. and then chilled to 4° C. The virus-antiserum mixture was inoculated into six volunteers and the virus control mixture into five volunteers. The results of this experiment are shown in Table 3. All those given virus alone were infected as shown by the re-isolation of virus or an antibody response. Four of the five subjects had symptoms or signs and one of the five had a cold. None of the six volunteers given the virus–antiserum mixture had laboratory evidence of infection, although one of them had symptoms and signs; these were insufficient to be regarded as a definite illness. This evidence suggested that the illnesses observed in all these experiments were due to para-influenza 2 virus.

DISCUSSION

It seems likely that the ability to re-isolate virus from volunteers 2 days or more after their intranasal inoculation was due to virus multiplication, since virus could not be re-isolated a few hours after inoculation. In several instances, virus was isolated from subjects with a respiratory illness and yet there was no evidence of an antibody response by any of the three serological techniques. This has been observed in natural and experimental infections with respiratory syncytial virus (Chanock, Kim, Vargosko, Deleva, Johnson, Cumming & Parrott, 1961; Kravetz, Knight, Chanock, Morris, Johnson, Rifkind & Utz, 1961; Hamre & Procknow, 1961) and in natural infections with para-influenza viruses (Chanock, Bell &Parrott, 1961). This stresses the point that a diagnosis of infection may have to be made on the evidence of virus isolation alone without an accompanying homotypic antibody response. The fact that we were not able to demonstrate heterotypic antibody responses by haemagglutination inhibition against other para-influenza viruses or mumps virus is perhaps not surprising; Heath, Tyrrell & Peto (1962) found that there was less correlation between the titres of haemagglutination-inhibiting antibody in human sera against CA virus and the other viruses of the para-influenza group than there was between the titres against para-influenza viruses 1 and 3.

It is difficult to explain the differences between our finding that satisfactory neutralization tests could be done with heated sera and the experience of Chanock (1956) who found that he could best demonstrate antibody responses if he used sera that were not inactivated by heating. The difference may have arisen because we used a different strain of virus and examined sera of adults rather than of children.

Tyrrell *et al.* (1959) found that para-influenza viruses 1 and 3 produced illnesses that fell within the range of illness produced by the rhinovirus H.G.P. in which coryza is a characteristic feature. The inoculation of volunteers with para-influenza 2 virus caused illnesses of a mild and somewhat different nature. Several volunteers had upper respiratory infections with nasal stuffiness, but without coryza, and therefore according to the criteria used at this unit these illnesses were not regarded as colds. Only four volunteers had illnesses with coryza which were

regarded as mild colds. Sore throat was quite a prominent feature of the illnesses and Mogabgab *et al.* (1961) noted this in adults from whom they isolated para-influenza 2 virus. The mild nature of the illnesses we observed might have been due to the fact that we used a virus that had had five passages in tissue culture. In one experiment, seven volunteers, all of whom possessed neutralizing antibody, were each inoculated intranasally with about 10 TCD 50 of virus which had not been passaged through tissue culture and which was contained in a throat washing. These volunteers were not infected, as judged by virus re-isolation or neutralizing antibody response, and they did not develop illness (Taylor-Robinson, unpublished). Although Tyrrell *et al.* (1959) showed that para-influenza 1 virus, which had only one passage in tissue culture, produced more infection and illness than virus that had had five passages, the severity of the illnesses was the same. It is possible that the apparently different nature of the illness produced by para-influenza 2 virus in adult volunteers is real. This would correspond with the difference noted in primary infection in children in whom para-influenza 2 virus has been shown to produce only croup, whereas para-influenza viruses 1 and 3 produce also bronchitis and pneumonia.

In our experiments, illness and infection occurred in the presence of pre-existing neutralizing antibody. This phenomenon has been observed in natural para-influenza infections of children and adults (Chanock *et al.* 1961) and in the experimental infections of adults with para-influenza viruses 1 and 3 mentioned previously. Because we found that there was no correlation between the presence of para-influenza 2 neutralizing antibody and the occurrence of illness it was important to determine whether the illnesses that we observed were due to para-influenza 2 virus or to some other agent, such as a simian virus 'picked up' by the passsage in monkey kidney cells. One of the subjects who was given para-influenza 2 virus that had been neutralized by specific antiserum prior to inoculation did present some symptoms and signs, but so did a few of the volunteers who had been given Hanks's saline. Further, there was a significantly greater number of symptoms, signs and illnesses in those who were inoculated with para-influenza 2 virus without antiserum than in subjects given the Hanks's saline and there were no illnesses in those volunteers who were not infected. We conclude from these facts that the para-influenza 2 virus caused the illnesses that we observed.

The results of these experiments seem to fulfil the third of Koch's postulates for para-influenza 2 virus as a cause of respiratory disease in adults. It must be pointed out, however, that we still know very little about how much and what sort of disease it causes under natural conditions.

SUMMARY

Twenty-eight adult volunteers were inoculated intranasally with para-influenza 2 virus and eight developed illnesses; twenty-eight volunteers were given Hanks's saline and one became ill. The illnesses occurred in volunteers given between 2×10^4 and 2×10^6 TCD 50 of virus. The most prominent symptoms were sore throat, nasal stuffiness and coryza; four of the eight volunteers had sufficient coryza to be regarded as having mild colds. Although only eight volunteers had

clinical evidence of infection, twenty-four had laboratory evidence of infection as judged by virus re-isolation or antibody response. Neutralization, haemagglutination-inhibition and complement-fixation tests on paired sera showed that sixteen individuals had a fourfold or greater antibody response by one or more tests including five of the eight volunteers who were ill. Twenty volunteers, including seven who were ill, had reciprocal neutralizing antibody titres of eight or more before inoculation of virus so it seems that the illnesses were due to re-infection in the presence of antibody. Evidence is presented which suggests that although illnesses occurred in the presence of antibody they were due to the para-influenza 2 virus and not some other agent in the inoculum. The results of these experiments seem to fulfil the third of Koch's postulates for para-influenza 2 virus as a cause of respiratory disease in adults.

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An outbreak of food poisoning caused by *Salmonella typhi-murium*, phage-type 12, probably spread by infected meat

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Not all outbreaks of salmonella food poisoning are explosive; some take the form of sporadic cases over a prolonged period of time. On inquiry, no common food and no single food premises are found to be implicated (Harvey & Phillips, 1961). Before the development of phage-typing of *Salmonella typhi-murium* (Felix & Callow, 1943; Callow, 1959; Anderson, 1960), many of the infections comprising such a group of related cases would have been regarded as sporadic. The use of phage-typing now enables the infections to be linked and allows them to be studied as a single episode instead of a series of apparently unrelated incidents of unknown origin (Anderson, Galbraith & Taylor, 1961). An outbreak caused by *S. typhi-murium*, phage-type 12, which occurred in South Wales in 1960, illustrates such a pattern of infection.

INVESTIGATION OF THE OUTBREAK

The human infections occurred over the period May to October 1960 (Table 1). The geographical distribution of the incidents is shown in Fig. 1. It will be noted that the main impact was experienced in Cardiff.

The patients were visited by staff of the City Health Department, and inquiries were made as to possible sources of infection. No single item of food could be implicated, but as meat was a possible cause of the outbreak, gauze swabs were placed in open drains in the abattoirs of Barry and Cardiff (Fig. 1). These drains were not subject to human pollution and it was considered that isolations of salmonellae from them would indicate the entry of infected animals into the abattoirs. The drains sampled received blood and faeces from the slaughtered animals. *S. typhi-murium*, phage-type 12, was isolated from the initial samples from both abattoirs and arrangements were made to receive regular specimens of drain swabs. Some samples of human sewage from Cardiff abattoir were also examined. The results are given in Table 1.

During the period of investigation, *S. typhi-murium*, phage-type 12, was isolated 14 times from Cardiff abattoir and 5 times from Barry abattoir. The prolonged

isolations of phage-type 12 from the Cardiff abattoir specimens over the 10-week period 8 July 1960 to 9 September 1960 is striking. In 5 years of abattoir swabbing, this is the only instance we have encountered of the isolation of the same salmonella strain from consecutive swabs for so long a period. The strain was isolated 14 times from drains receiving material from pig slaughter and 13 times from drains receiving material from cattle slaughter. The human sewage from Cardiff abattoir was sampled regularly from the week ending 22 July 1960 and was positive on

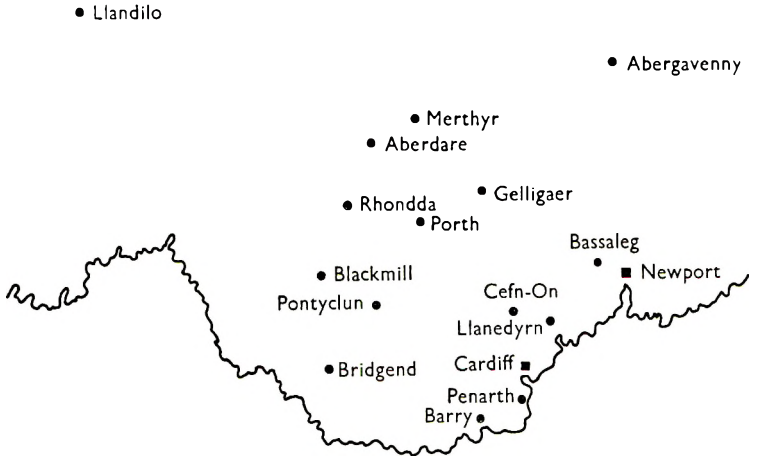

Fig. 1. Geographical distribution of human and animal incidents caused by *S. typhi-murium*, phage-type 12, in South Wales.

Human incidents		Animal incidents on farms		
Place	No. of incidents	Place	Date	Animals infected
Aberdare	1	Llandilo	12 October 1959	Pigs
Barry	4	Abergavenny	30 December 1959	Pigs
Blackmill	2	Bassaleg	7 July 1960	Cows
Bridgend	1	Llanedyrn	18 July 1960	Pigs
Cardiff	81			
Cefn-On	1			
Gelligaer	2			
Merthyr	2			
Penarth	6			
Pontyclun	1			
Porth	2			
Rhondda	2			
	105			

Total human infections = 122
Sporadic cases = 95
Family incidents = 10

six occasions for phage-type 12. As the view is sometimes put forward that salmonella isolations from an abattoir may have a human source, the sampling of human sewage at Cardiff slaughter house was regularly continued after the end of the outbreak. This sewage was last found positive at the abattoir in the week

Table 1. *Time relations in the outbreak*, 1960

Month ...	May			June				July					August				September					October				November			
Week ending ...	13	20	27	3	10	17	24	1	8	15	22	29	5	12	19	26	2	9	16	23	30	7	14	21	28	4	11	18	25
Human incidents, Glamorgan	1	0	0	4	2	11	12	16	21	16	6	3	2	2	3	2	0	0	1	0	0	1	2	0	0	0	0	0	0
Cardiff abattoir, drains receiving material from slaughter	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	−	+	−	−	.	+
Cardiff abattoir, human sewage	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	−	+	−	−	.	−	−	−	−	−	−	−	.	−
Cardiff abattoir, faeces of staff, 63 persons*	.	.	.	.	.	.	.	.	.	.	+ 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cardiff abattoir, pig faeces, 69 pigs*	.	.	.	.	.	.	.	.	.	.	+ 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cardiff abattoir rats; only one rat caught*	.	.	.	.	.	.	.	.	.	.	+ 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Retail shop drains, Cardiff	.	.	.	.	.	.	.	.	.	+	+	+	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.
Retail shops, Cardiff food handlers, 112 persons*	.	.	.	.	.	.	.	.	.	.	+ 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Barry abattoir, drains receiving material from slaughter	.	.	.	.	.	.	.	.	.	+	−	+	+	+	−	+	−	−	−	−	−	−	−	−	.	−	−	−	−

* Figure underneath + sign denotes number of animals or persons found positive.

ending 2 September 1960. Since then it has not once been positive over a period of 30 months, although frequent isolations of salmonellae have been made from drains receiving material from slaughtered animals. It is thus improbable that the salmonella strains isolated from the abattoir had a human origin. The technique of examination was that described previously (Harvey & Phillips, 1961).

From the records of the Veterinary Investigation Department, Cardiff, it was found that type 12 had been isolated from animals on three farms in South Wales in 1959–60. The isolations had been made twice from pigs and once from a cow. It was arranged during the outbreak to examine individual cow and pig faeces taken at the city abattoir. Altogether 201 cattle faeces and sixty-nine pig faeces were examined. All the cattle faeces were negative, but one pig faeces was found to be positive for *S. typhi-murium*, phage-type 12. The pig from which the positive specimen was obtained was traced to a farm at Llanedyrn—a village between Cardiff and Newport. The geographical position of all animal incidents on farms is given in Fig. 1.

One rat was caught in the Cardiff abattoir. *S. typhi-murium*, phage-type 12, was isolated from it.

Table 2. *Isolation of* Salmonella typhi-murium, *phage-type* 12—*food premises*

Food premises no.	14 July	18–19 July	22 July	26–27 July	31 July	17 Aug.	25 Aug.	Later	Description of retail premises
1	−	+	.	−	.	.	.	−	Butcher
2	+	−	.	−	.	.	.	−	Butcher
3	+	+	.	−	.	.	.	−	Butcher
4	.	+	.	−	−	.	.	.	Butcher
5	−	+	.	−	.	.	.	−	Butcher
6	+	+	−	.	.	.	.	−	Butcher
7	+	+	+	.	.	.	.	−	Butcher
8	+	.	+	−	.	.	.	−	Butcher
9	+	−	.	−	.	.	.	−	Butcher
10	+	+	.	+	.	.	.	−	Butcher
11	+	+	.	−	.	.	.	−	Butcher
12	+	−	.	−	.	.	.	−	Baker
13	+	−	.	−	.	.	.	−	Butcher
14	.	.	.	−	.	−	−	+	Butcher
15*	.	−	.	−	.	.	.	.	Butcher
16	−	+	.	−	.	.	.	−	Baker

* Drains receiving material from food preparation rooms negative, but butcher found to be excreting *S. typhi-murium*, phage-type 12.

In a previous group of related cases due to *S. typhi-murium*, 1 a var. 3 (Harvey & Phillips, 1961), it had been possible to isolate the organism from farm animals in South Wales, from abattoir samples and from local human infections. In the present outbreak, we wished to follow the path of infection to retail food premises. As most of the human incidents occurred in Cardiff, it was decided to examine food shops in the city. The sampling technique used was identical with that previously employed in the drains of abattoirs.

The shops examined were selected either because they were directly associated with notified cases, or because they were located in the neighbourhood of groups of cases. They comprised fifty-four premises of mixed types.

Swabbing was carried out from July to September 1960. *S. typhi-murium*, phage-type 12, was isolated by the drain-swab technique from thirteen butchers' shops and two bakehouses. The latter premises sold made-up meat products in the form of sausage rolls as well as confectionery. Both bakehouses belonged to the same firm. Although the organism was not isolated by drain sampling from a further butcher's shop, one of the employees at this shop was found to be excreting phage-type 12. This man was also employed as an assistant slaughterman at the Cardiff abattoir. It was possible, therefore, to demonstrate the presence of the type on the premises of 16/54 of the selected food establishments. Details of the premises in which the organism was found are given in Table 2.

Other strains of salmonellae were also isolated from these food premises, namely *S. typhi-murium*, phage-types 2c, 1, 1a var. 3 and untypable; *S. thompson*, *S. give*, *S. kiambu*, *S. derby*, *S. coley-park*, *S. binza*, *S. brandenburg*. All these isolations were made from premises dealing in raw or processed meat.

Faecal swabs were taken from all the staff of the infected food premises (total 112). Of these employees, only one was positive—the man mentioned previously who was also an apprentice slaughterman at the abattoir (Table 2, food premises no. 15). This man was clear of infection on 10 August 1960.

SUMMARY

An outbreak of food poisoning caused by *Salmonella typhi-murium*, phage-type 12, in which 122 persons were infected, is described.

Isolation of the same organism from the drains of two large abattoirs, from pig faeces at one abattoir, and from the floor drains of fifteen out of fifty-four butchers' shops and bakehouses examined, together with records showing that this organism had been recently isolated from pigs and cattle in the neighbourhood, suggested that meat was the vehicle of infection.

We are indebted to Dr E. S. Anderson and the staff of the Central Enteric Reference Laboratory and Bureau, Colindale, for phage-typing the cultures of *S. typhi-murium*.

We should like to thank Prof. Scott Thomson for his encouragement and advice in the preparation of this paper and Mr J. Morgan and Mr B. M. Jones of the Public Health Laboratory, Cardiff, for their technical assistance.

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A trial of reinforcing doses of diphtheria toxoid absorbed through the buccal mucosa

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The administration of diphtheria toxoid without injection has been tried by nasal instillation (Jensen, 1937), inhalation (Bousfield & King-Brown, 1938), application to the skin (Fraser, Davey & Halpern, 1940; Bousfield, 1944) and orally in a tablet which was swallowed (Bousfield, 1945; Greenberg & Fleming, 1950). These methods were either ineffective or gave rise to serious side-effects (Lancet, 1941) which precluded their use. Bousfield (1945) and Masucci, Gold & DeFalco (1948) showed that toxoid administered in a lozenge which was allowed to dissolve slowly in the mouth was free from side-effects and produced an antitoxin response in most persons with a history of previous immunization or an attack of diphtheria. Cockburn and his colleagues, in a preliminary study using lozenges containing diphtheria toxoid and hyaluronidase, demonstrated a satisfactory antitoxin response in a small group of adults (Cockburn, Bradstreet, Bailey & Ungar, 1961).

The purpose of this trial was to find out whether these lozenges were a satisfactory means of reinforcing immunity in children aged 10–12 years who had been previously immunized by parenteral injection, and to compare the antitoxin response with that following the injection of formol toxoid.

MATERIALS AND METHODS

The children

The trial took place in three schools in Manchester. The children were between 10 and 12 years old and had records of primary immunization with at least two injections of diphtheria prophylactic with or without subsequent reinforcing doses, the last dose having been given more than 12 months before the commencement of the trial.

One-hundred and eighty children took part in the trial and were divided into three groups of sixty each by means of random sampling numbers. One group received formol toxoid by injection; another group two lozenges at an interval of

* The trial was designed and the results analysed by Dr W. C. Cockburn (present address: Division of Communicable Diseases, World Health Organisation, Geneva) and Dr N. S. Galbraith, Central Public Health Laboratory, Colindale, London, N.W. 9. Dr C. Metcalfe Brown, Dr E. M. Jenkins, Dr M. C. Davitt, Dr S. J. Jaron and Dr J. M. McCarthy, Manchester, carried out the field investigations. The antitoxin titrations were performed by Dr C. M. P. Bradstreet and Miss E. M. Bailey, Colindale. The diphtheria toxoid lozenges were prepared by Dr J. Ungar, Glaxo Research Laboratories.

1 week; and the third group three lozenges, the first two at an interval of 1 week and the third 1 month later.

Blood specimens were collected from the children immediately before the administration of the toxoid and again between 2 and 3 weeks after the injection or the last lozenge. One year later a third blood specimen was obtained from as many children as possible whose initial antitoxin titres were equal to or less than 0·1 unit per ml. and also from a sample of children in the lozenge groups whose titres were between 0·1 and 1·0 unit per ml.

The toxoid

Each lozenge contained 1500 Lf. of diphtheria toxoid and 1500 units of hyaluronidase (Cockburn *et al.* 1961). The lozenges were given to the children at school under supervision and the children were instructed to allow the lozenges to dissolve slowly in their mouths.

The formol toxoid, in a dose of 25 Lf., was given by intramuscular injection.

The antitoxin titrations

The blood samples were centrifuged within 24 hr. after collection and the sera were stored at −20° C. until required. The titrations were done on the paired samples when all the second sera had been collected. The titrations of the second sera were repeated with the corresponding third sera in the children who were bled again 1 year after immunization. The antitoxin was titrated by the method previously described (Cockburn *et al.* 1961).

Table 1. *Number of children immunized in the trial and number of sera examined*

Immunization group	Number of children: Immunized	First and second sera examined	Selected for bleeding 1 year after immunization	Third sera examined
Injection	60	46	20	12
Two-lozenge	60	52	68	44
Three-lozenge	60	36		
Total	180	134	88	56

RESULTS

One hundred and eighty children were included in the trial, and paired blood samples were examined from 134 (Table 1). In forty children paired sera were not obtained; in two the serum containers broke in transit; in one the serum control gave a positive result; in one the guinea-pig died and there was insufficient serum to repeat the test; and in two children with the same name and initials the specimens could not be distinguished.

Of the 134 remaining children, 67 were boys and 67 girls; 46 children received formol toxoid by injection, 52 received two lozenges and 36 received three lozenges.

Eighty-eight of the children were selected for bleeding 1 year after immunization, and sera were obtained from 56 of them (Table 1). There were no reports of any reactions after the administration of the lozenges and the children did not find them unpleasant to take.

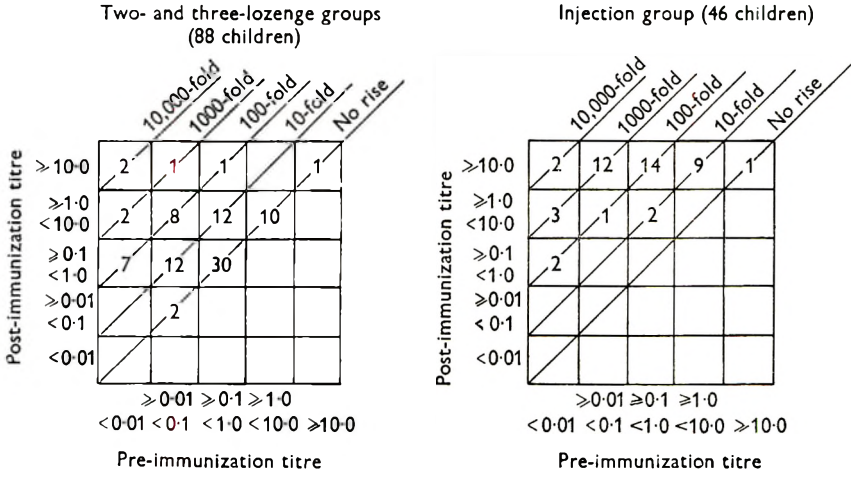

Fig. 1. Antitoxin titres before and 2–3 weeks after immunization.

Table 2. *Antitoxin titres in fifty-six children from whom serum samples were obtained 1 year after immunization*

Immunization group and number immunized	Serum sample	Antitoxin titre (units per ml.) < 0·01	⩾ 0·01 < 0·1	⩾ 0·1 < 1·0	⩾ 1·0 < 10·0	⩾10·0
		Number of children				
Injection (12 children)	Pre-immunization	4	7	1	0	0
	2–3 weeks after immunization	0	0	1	3	8
	1 year after immunization	0	1	4	6	1
Two-lozenge and three-lozenge (44 children)	Pre-immunization	7	15	22	0	0
	2–3 weeks after immunization	0	1	25	15	3
	1 year after immunization	0	7	30	5	2

Vertical line indicates antitoxin titre below which immunization is thought to be necessary (Barr & Cunliffe, 1954).

Fig. 1 shows the antitoxin titres before and 2–3 weeks after immunization in the three groups of children. As the antitoxin response in the two-lozenge and three-lozenge groups was similar they have been combined in the figure. Of eleven children with initial titres of less than 0·01 unit per ml., all had titres of 0·1 unit or more after immunization. Only two of thirty-four children with initial titres less than 0·1 unit failed to respond to immunization by lozenge.

The antitoxin response in the injection group was greater than in the lozenge groups. There was no difference in the antitoxin response between boys and girls or between children from different schools. The antitoxin response was greater in children with low initial titres than in those with high titres. This difference was more noticeable in the lozenge groups than in the injection group.

The antitoxin titres in fifty-six children from whom a blood sample was obtained 1 year after immunization are shown in Table 2. In the injection and the lozenge groups the titres had fallen after 1 year, but in all the children the titres were equal to or greater than 0·01 unit per ml. Seventeen of the children tested in the lozenge groups with initial titres greater than 0·01 unit per ml. had no rise in titre after immunization; none of them showed any rise a year later, but in none did the titre fall below 0·01 unit.

DISCUSSION

The Schick test, which is often used to determine immunity or susceptibility to diphtheria, does not correspond to a precise antitoxin titre, but is usually negative when the titre is greater than 0·004 unit per ml. and positive below this (Greenberg & Roblin, 1949; Barr, Stamm & Stevens, 1957). Most of the children in the trial would probably have been Schick negative before immunization; after immunization they would all have been Schick negative, as none of them had post-immunization titres of less than 0·01 unit per ml.

Barr & Cunliffe (1954) and Barr *et al.* (1957) considered that the Schick test titre of antitoxin was not satisfactory as a measure of immunity in an artificially immunized community, and suggested that a titre of less than 0·01 unit per ml. indicated that further immunization was necessary.

Eleven children in the lozenge groups had initial titres of less than 0·01 unit and all had a 100-fold or greater rise in titre after immunization (Fig. 1). Thirty-two (94 %) of the thirty-four children in lozenge groups with initial titres below 0·1 unit had an antitoxin response. It appears, therefore, that the lozenges were a satisfactory means of reinforcing immunity in this group of 10–12 year old children.

There was little difference in the response of the two-lozenge and three-lozenge groups. The response in both was less than in the injection group, but the lozenges produced a substantial rise in antitoxin titre in children with low initial titres.

The oral route has considerable administrative advantages over injection, and children would probably accept oral immunization more readily. The results of this trial suggest that, in children who have been previously immunized by injection, the reinforcing injection usually given between 10 and 12 years of age might be replaced by a course of two lozenges at an interval of 1 week. The lozenges might also be a valuable means of reinforcing the immunity of a large number of children quickly during an epidemic of diphtheria.

The lozenges must be administered under supervision to ensure that the children allow them to dissolve slowly in their mouths. If they are swallowed they are likely to be ineffective.

SUMMARY

Diphtheria toxoid lozenges were given to eighty-eight children aged 10–12 years who had previously been immunized by parenteral injection, and the antitoxin titres before and after administration were compared with those in a similar group of forty-six children who received formol toxoid by injection.

The results showed that a course of two lozenges at an interval of 1 week was a satisfactory means of reinforcing immunity in the children. The antitoxin response was less than that after injection, but a substantial response occurred in the children with low initial titres.

It is suggested that the lozenges might replace an injection as a means of reinforcing immunity in children aged 10–12 years and that they might be a valuable means of rapidly reinforcing the immunity of a large number of children in an epidemic.

The lozenges must be given under supervision to ensure that the children allow them to dissolve slowly in their mouths.

We should like to thank Dr J. D. Abbott of the Public Health Laboratory, Manchester, for separating the sera.

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With 1 *plate*
Printed in Great Britain

The production and inactivation of pyocines

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(*Received* 23 *May* 1963)

Most strains of *Pseudomonas aeruginosa* produce substances known as pyocines which inhibit the growth of other strains of the same species. Pyocines, first described by Jacob (1954) and studied in detail by Hamon (1956), belong to the class of substances known as bacteriocines (Jacob, Lwoff, Siminovitch & Wollman, 1953) and the fact that they are active only on closely related organisms differentiates them from the many other antibiotic substances produced by *Ps. aeruginosa* (Emmerich & Löw, 1899; Schoental, 1941; Hays *et al.* 1945; Young, 1947). There is considerable variation in pyocine activity of different strains within the species and it has been suggested that this could form the basis of a typing scheme (Holloway, 1960; Papavassiliou, 1961). It was clear, however, from preliminary experiments in this laboratory that such a scheme could not be employed until a more reliable method of testing for pyocine production had been devised. The degree of inhibition of one strain by another varied with the medium used and other cultural conditions. Several strains which had been described by other workers as non-producers of pyocines were found to be active. It was also noted, in cross-streaking experiments, that an area of growth of the indicator strain often occurred in the centre of a zone of inhibition, suggesting that *Ps. aeruginosa* strains produced, as well as pyocines, other substances which antagonized their action. Brubaker & Surgalla (1961) made similar observations on an inhibitory substance while investigating the pesticines produced by *Pasteurella pestis*.

This work describes experiments to determine the optimum conditions for the production and demonstration of pyocines and the nature of the pyocine-inactivating substances.

MATERIALS AND METHODS

Strains

Pseudomonas aeruginosa

(*a*) Producing pyocines: N.C.T.C. 1999, B 55, M 163, S 178 and H 194. Although all these strains had different ranges of activity when tested on a large series of indicator strains, the first four of them inhibited all four indicator strains listed under (*c*); strain H 194, however, inhibited only the indicator strain M 8.

(*b*) not producing pyocines: W 96 and P 501. These two strains did not inhibit any of a large series of indicator strains including those listed under (*c*).

(*c*) Indicator strains: M 8, B 10, S 17 and B 26. The first of these was inhibited by all the strains listed under (*a*) and the other three were inhibited by all except strain H 194.

Proteus vulgaris

A non-motile strain (N.C.T.C. 8313) was used for the production of bacterial proteinase.

Media

(*a*) Nutrient broth: (Oxoid no. 2) containing 1% 'Lab Lemco' beef extract, 1% peptone (Oxoid L 37), 0·5% sodium chloride pH 7·5.

(*b*) Nutrient agar (Oxoid no. 2) containing similar ingredients to the nutrient broth, plus 1% 'Ionagar' no. 2 (Oxoid) pH 7·5.

Technique

The procedure used in most of the experiments was similar to that employed by Abbott & Shannon (1958) in studying *Shigella sonnei* colicine production.

Thick nutrient agar plates (8–10 mm. deep) were inoculated with the strain under test as a central streak 1 cm. wide, and incubated at 37° C. for varying periods of time. After incubation, the plates were exposed to chloroform vapour for 1 hr. and the growth was then scraped off with a microscope slide. The plates were re-exposed to chloroform for 1 hr. and left for a further hour with their lids off, to allow the chloroform to evaporate. Four-hour nutrient broth cultures of each of the four indicator strains were then streaked across each plate at right angles to the line of the original inoculum. After re-incubation for 16 hr. the plates were examined for evidence of inhibition of growth of the indicator strains. Any deviation from this technique is reported in the text.

EXPERIMENTAL

(1) *Formation of pyocines on solid media*

Five nutrient agar plates were inoculated with strain N.C.T.C. 1999, and incubated for 16, 24, 36, 48 and 72 hr., respectively. After killing and removing the growth, each plate was inoculated with the four indicator strains and re-incubated. The width of the zone of inhibition of each indicator strain on each plate is shown in Text-fig. 1. Large zones appeared on the plate on which the pyocinogenic strain had been growing for 24 hr. On the plate incubated for 36 hr., however, the zones were distinctly smaller, though they were again larger on the plates incubated for 48 and 72 hr. This suggested that a period of production of pyocines was followed by a period in which inactivating substances were produced.

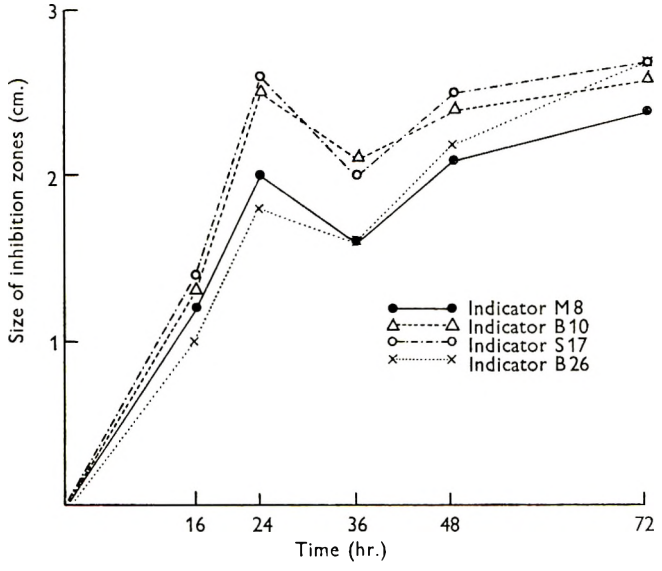
When incubation was done for periods longer than 72 hr. (up to 7 days) zones of inhibition of a more or less constant width were obtained.

The same experiment was repeated with strains B 55 and S 178 and similar results were obtained. The reduction in zone size after the 24 hr. period differed slightly with the different pyocinogenic strains, but was always demonstrated.

(2) *Demonstration of the pyocine-inactivating substances*

(*a*) A plate was inoculated with strain N.C.T.C. 1999 in the usual manner and incubated for 24 hr. After the growth had been killed and scraped off, the same area was re-inoculated with the non-pyocine-producing strain W 96. After 24 hr.

re-incubation, the resulting growth was killed and removed. The indicator strains were then inoculated at right angles to the original streaks and the plate was examined after a further 16 hr. incubation. No inhibition of the indicator strains was seen. A control plate, prepared in the same way but not inoculated with strain W 96, showed the usual zones of inhibition, indicating that strain W 96 produced substances which inactivated the pyocines elaborated by strain N.C.T.C. 1999.



Text-fig. 1. Zones of inhibition of four indicator strains produced after different periods of growth of the pyocinogenic strain N.C.T.C. 1999.

(*b*) A streak of strain M 163, 2 cm. wide, was made to one side of the middle line of a 14 cm. nutrient agar plate (streak A, Pl. 1, fig. 1). After 48 hr. incubation, the growth was sterilized and removed. Strain P 501 was then inoculated in a similar manner on the other side of the middle line, in the upper half of the plate only (streak B). After a further 48 hr. incubation this growth was killed and removed, and the four indicator strains were each streaked across the plate, once on the upper half of the plate and again on the lower half. Pl. 1, fig. 1 shows the result of this experiment. On the upper half of the plate there was no inhibition of growth of the indicator strains on the side on which strain P 501 had grown, suggesting that this strain produced pyocine-inactivating substances but no pyocines. On the side on which M 163 had been grown, however, the usual inhibition of the indicators occurred. In the lower control half of the plate the zone of inhibition extended symmetrically on both sides of the primary streak of strain M 163.

(*c*) Strains N.C.T.C. 1999, B 55 and S 178 were stabbed with a straight wire into a nutrient agar plate at 3 points on a horizontal line 2 cm. apart. After 48 hr. incubation, the resulting macrocolonies were killed by chloroform and scraped off. Strain W 96 was then heavily streaked on the plate, parallel to the previous line and at a distance of 2 cm. from it. After another 48 hr. incubation, the resulting growth was killed and removed, and the plate flooded with a 4 hr. broth culture

of indicator strain M 8. Pl. 1, fig. 2 shows this plate after 16 hr. re-incubation. The effect of the pyocines produced by strains B 55 and S 178 appears as larger inhibition zones of the indicator strain on the side of the macrocolonies away from the line where strain W 96 had been grown, than on the other side. With strain N.C.T.C. 1999, there is no zone of inhibition. It was concluded that the pyocines produced by strains B 55 and S 178 were partially inactivated by substances derived from strain W 96 and that produced by N.C.T.C. 1999 was completely inactivated.

(3) *Effect of proteolytic enzymes on pyocines*

(*a*) Strains N.C.T.C. 1999, B 55, M 163 and S 178 were stabbed with a straight wire into a nutrient agar plate in two rows about 4 cm. apart. After 48 hr. incubation, followed by exposure to chloroform and then air, a strip of thick sterile blotting paper (1 × 7 cm.) soaked in a 1:1000 solution of crystalline trypsin was placed in the central area between the macrocolonies. The plate was then incubated for 2 hr., the paper was removed, and the plate flooded with a 4 hr. nutrient broth culture of the indicator strain M 8. After 16 hr. re-incubation, it was found that there was no inhibition of the indicator strain by strains N.C.T.C. 1999 and B 55, since their pyocines had been completely inactivated. With strain M 163, however, slight inhibition of the indicator strain occurred as a result of partial inactivation of pyocine, while with strain S 178 normal inhibition occurred as the pyocine had not been affected. The results suggested that the pyocines produced by different strains varied in their susceptibility to the inactivating action of trypsin.

(*b*) A thick sterile blotting paper strip was soaked in trypsin solution and placed to one side of the middle line of a nutrient agar plate. A thin layer of agar was then poured over the whole surface of the plate. When this had solidified, strain B 55 was inoculated over the blotting paper area and to a distance of 1 cm. to one side of it. After 24 hr. incubation, the growth was killed and removed, and the plate then flooded with a 4 hr. nutrient broth culture of the indicator strain M 8. After a further 16 hr. incubation, the pyocinogenic activity of strain B 55 could only be demonstrated by an ill-defined zone of inhibition, away from the blotting paper area, indicating the ability of trypsin to inactivate pyocines.

(*c*) The previous two experiments were repeated, replacing the trypsin with a 20 % solution of papain in 0·2 M phosphate buffer containing 0·001 M versene and 0·005 M cysteine. Inactivation of pyocines by papain could not be demonstrated.

(4) *Effect of a bacterial proteinase*

A set of experiments similar to 2*b* and 3*a* was performed, replacing the strain W 96 and the blotting paper strip respectively by a streak of the *Proteus vulgaris* strain N.C.T.C. 8313, and incubating the plates for 48 hr. The rest of the procedures were then followed as before. The results showed that this strain of *Proteus* completely inactivated the pyocines produced by strains N.C.T.C. 1999 and B 55. Those pyocines produced by strain M 163 were partially inactivated and those produced by strain S 178 remained unaffected. These results were similar to those obtained using trypsin and suggest that the effect of the *Proteus* on pyocines may be due to proteinase action.

(5) *Passage of pyocines through membrane filters*

Membrane filters (Oxoid size 4 cm., porosity 0·5–1·0 μ) were placed in the centre of three nutrient agar plates. Strain N.C.T.C. 1999 was inoculated in a $\frac{1}{2}$ cm. wide band across the filter. After 24, 48 and 72 hr. incubation, respectively, the filters were removed and 4 hr. nutrient broth cultures of the four indicator strains streaked on the area previously occupied by the membrane filter and at right angles to the position of the original streak. After another 16 hr. incubation, growth and inhibition zones were recorded. Strain N.C.T.C. 1999 was selected for this experiment since excessively motile strains tended to grow over the edge of the membrane filter within 48 hr.

Inhibition of indicator strain B 10 appeared after 48 hr. but was not detected at 24 or 72 hr. The other indicator strains were not inhibited at any time. From these results it was concluded that only some pyocines were able to pass through the membrane filter. The fact that pyocines could be detected after 48 hr., but not later, suggested that the inactivating substances were formed later than the pyocines and also passed through the membrane. The inability to demonstrate pyocines affecting indicator strains other than B 10 suggested the simultaneous passage of certain pyocines and their inactivating substances through the membrane.

(6) *Effect of the composition of the medium on the production of pyocines and the suppression of the inactivating substances*

A common finding in cross-streaking experiments was the appearance of discrete colonies or central growth in the inhibition zones of the indicator strains. This interfered with the proper reading of these inhibition zones. The colonies were repeatedly tested and found sensitive to the pyocine-producing strains with very rare exceptions, suggesting the involvement of a mechanism different from resistance. When the presence of pyocine-inactivating substances was demonstrated in the previous experiments, a trial was made for the suppression of the central growth.

The following substances were added either alone or in various combinations to the basal nutrient agar medium:

Calcium chloride	0·1 M	Sodium citrate	0·1 %
Manganese chloride	0·01 M	K_2HPO_4	0·1 %
Cupric sulphate	10^{-6} M	Protamine sulphate	1 %
Iodoacetic acid	10^{-5} M	Ethylenediamine tetra-acetic acid	1 %

Strain B 55 was inoculated by the streak technique on to plates containing each medium. Primary incubation was for 48 hr. and after inoculation of the four indicator strains, further incubation was for 16 hr. The inhibition zones were measured and any appearance of discrete colonies or confluent growth in the centre of the zones was recorded. A control nutrient agar plate was included in the series.

From Table 1 it can be seen that the addition of cupric sulphate increased the size of the inhibition zones of indicator strains M 8 and S 17, suggesting that this

substance stimulated the production of particular pyocines. Calcium chloride and manganese chloride had a small enhancing effect on the production of pyocines but there was apparently a simultaneous increase in the activity of the inactivating substances, as discrete colonies and even confluent central growth appeared in the inhibition zones. Sodium citrate and dipotassium hydrogen phosphate did not increase the inhibition zones but suppressed the appearance of any colonies in the zone. Another advantage of the medium containing citrate and phosphate was the absence of slime production. It had been observed that when a strain of *Ps. aeruginosa* formed abundant slime it was difficult to remove all the growth from

Table 1. *Effect of eight substances on the inhibition zones produced by pyocinogenic strain B* 55 *on the indicator strains M* 8, *B* 10, *S* 17 *and B* 26

(Zone sizes expressed in cm.)

Substance	Concentration used	Indicator strains M 8	B 10	S 17	B 26
Cupric sulphate	10^{-6} M	3·4	2·0	3·6	2·0
Calcium chloride	0·1 M	1·8c	2·0g	2·0g	2·0g
Manganese chloride	0·01 M	1·7c	1·8c	2·0c	1·8c
Sodium citrate	0·1 %	1·4	1·5	1·6	1·8
Dipotassium hydrogen phosphate	0·1 %	1·5	1·4	1·5	1·8
Iodoacetic acid	10^{-5} M	1·4	1·4	1·8	2·0
Protamine sulphate	1 %	1·5	1·3	1·6	1·8
EDTA	1 %	1·4c	1·4c	1·6	1·9c
Combination of citrate, phosphate and iodoacetic acid	—	1·5	1·4	1·7	2·0
Control (nutrient agar)	—	1·5c	1·5c	1·7	1·8

c = discrete colonies in centre of inhibition zone.
g = confluent growth in centre of inhibition zone.

the plate by scraping, and the remnants of slime sometimes caused an irregular deposition of the indicator strain on the plate. Iodoacetic acid and protamine sulphate had an effect similar to that of citrate and phosphate, and resulted in continuous, well-defined inhibition zones. Ethylenediamine tetra-acetic acid (EDTA) did not affect the inhibition zones and did not diminish the appearance of growth in the zones.

The medium finally adopted for examining pyocine production contained 0·1 % sodium citrate, 0·1 % dipotassium hydrogen phosphate and 10^{-5} M iodoacetic acid.

(7) *Effect of temperature on the stability of pyocines*

Twelve nutrient agar plates were inoculated by the streak technique with strain N.C.T.C. 1999 and incubated for 48 hr., and then divided into three sets of four plates each. The first set was kept in the incubator at 37° C., the second at room temperature (approx. 22° C.) and the third at 5° C. One plate from each set was tested with the four indicator strains after 1, 2, 3 and 6 days. Inhibition zones and appearance of isolated colonies or confluent central growth were recorded (Table 2). The size of the zones and the appearance of isolated colonies was not influenced by storage at 5° C., or at room temperature for periods up to 6 days.

On the plates kept at 37° C., the size of the zones was also unaffected, but confluent central growth started to appear on the second day with indicator strains B 10 and B 26, suggesting an increased action of the inactivating substances at 37° C.

Table 2. *Effect of the storage temperature on the pyocines produced by strain N.C.T.C.* 1999 *as assayed by the inhibition zones on four indicator strains*

(Zone sizes expressed in cm.)

Storage conditions	Indicator strains M 8	B 10	S 17	B 26
Incubator 1 day	1·5	1·4	1·5	1·6
2 days	1·3	1·1g	1·4	1·5g
3 days	1·3	1·5g	1·6	1·3g
6 days	1·4	1·4g	1·4	1·7g
Room temperature 1 day	1·5	1·4	1·5	1·5
2 days	1·3	1·4c	1·4	1·5c
3 days	1·4	1·4	1·7	1·4c
6 days	1·6	1·4c	1·7	1·5
Cold room 1 day	1·2	1·1	1·4	1·3
2 days	1·3	1·3c	1·4	1·3c
3 days	1·4	1·7	1·5	1·4
6 days	1·3	1·4	1·4	1·3

c = discrete colonies in centre of inhibition zone.
g = confluent growth in centre of inhibition zone.

Table 3. *Effect of fifty subcultures on the production of pyocines of strains N.C.T.C.* 1999, *B* 55 *and H* 194 *as assessed by the inhibition zones on four indicator strains*

(Zone sizes expressed in cm.)

Pyocinogenic strain		Indicator strains M 8	B 10	S 17	B 26
N.C.T.C. 1999	before subculture	1·6	1·4	1·6	1·5
	after 50 subcultures	1·5	1·5	1·4	1·6
B 55	before subculture	1·8	1·9	2·0	1·9
	after 50 subcultures	1·9	1·7	2·0	1·6
H 194	before subculture	0·7	—	—	—
	after 50 subcultures	0·8	—	—	—

(8) *Effect of frequent subculture and prolonged storage on the production of pyocines*

It has been claimed that *Ps. aeruginosa* strains lose their ability to produce pyocines on storage (Hamon, Véron & Péron, 1961). Three pyocinogenic strains, two with a wide range of activity (N.C.T.C. 1999 and B 55) and one with a narrow range (H 194) were used in the following experiment. Each was subcultured 50 times over a period of 3 months both on nutrient agar slopes and in broth tubes and tested for the production of pyocines every 10 subcultures by the usual

cross-streaking technique with the four indicator strains. Inhibition zones remained constant throughout the experiment (Table 3).

Other strains which were known to have been kept for long periods under unfavourable cultural conditions were also examined; they included 10 strains which had been stored on nutrient agar slopes at room temperature for 5–8 years, with 6- to 12-monthly subcultures. All were good producers of pyocines and their patterns were similar to those of freshly isolated strains.

DISCUSSION

In studying the production of bacteriocines by *Shigella sonnei* by the cross-streaking method, Abbott & Shannon (1958) noted that discrete colonies, or even confluent growth, often appeared in the centre of the zone of inhibition of an indicator strain. They named these appearances 'D zones', and considered them to be due to the growth of a minority of organisms that were resistant to the bacteriocine. On testing the sensitivity of these colonies in comparison with the original culture it was found, however, that they were rarely truly resistant (Abbott, pers. com.). Similar findings have now been obtained with *Pseudomonas aeruginosa* cultures. The results suggest that the pyocine, which is first formed at the centre of the primary streak and diffuses outwards, is inactivated by substances which are produced at a later stage of the growth cycle. At the periphery of the growing pyocinogenic strain, where the inactivating substances have not yet been produced, the indicator strains are inhibited by the pyocines formed early in the growth phase. The motility of a particular pyocinogenic strain may also play a part in the formation of the D zones, as an actively motile strain will tend to move the bilateral area of inhibition further towards the periphery. It has been demonstrated that this phenomenon was not simply due to the diffusion of the pyocines into the periphery; diffusion alone would have resulted in a gradual increase in zone size with or without central growth of the indicator strains, but in fact the zone size decreased between the 24 and 48 hr. periods. This could only be explained by the appearance of some other substances interfering with the activity of the pyocines. The appearance of 'resistant' colonies, or confluent control growth, is therefore due to the inactivation of the pyocines. When there is little pyocine inactivation, a few discrete colonies will appear, but when most of the pyocines have been inactivated, confluent growth (D zones) is obtained. Further, it has been shown that different pyocines vary in their susceptibility to the inactivating substances. It is probable that much of the difficulty experienced by some workers in demonstrating the pyocinogenic activity of some strains of *Ps. aeruginosa* may have been due to simultaneous production of pyocine-inactivating substances.

The presence of pyocine-inactivating substances was also demonstrated by the membrane filter experiment, where pyocines were detected on the second day but not on the third, suggesting that the pyocines passed the membrane first and the inactivating substances later. Their presence can also explain the curves in Text-fig. 1, where production of pyocines occurs first, followed at a later stage by the inactivating substances. This resembles the formation and subsequent

destruction by bacterial proteases of staphylokinase (Elek, 1953) and of staphylococcal coagulase (Lominski, Smith & Morrison, 1953).

Gratia & Betz-Bareau (1946) and Frédéricq (1948) reported that colicines produced by coliform bacilli were inactivated by trypsin and bacterial proteinases. According to Hamon (1956), pyocines are also protein in nature. The experiments described here, in which pyocines were inactivated similarly by strains of *Ps. aeruginosa*, a strain of *Proteus vulgaris*, and the proteolytic enzyme trypsin, provide some evidence that the inactivating substances were proteinases. It was also shown that calcium, which enhances the proteinases of pseudomonas and proteus cultures (Haines, 1932), has a similar effect on the pyocine inhibitors. Hamon & Péron (1962) found that some of the bacteriocines of *Ps. aeruginosa* were resistant and others sensitive to the action of trypsin.

Maschmann (1937) showed that strains of *Ps. aeruginosa* produced a wide variety of different proteinases and used phosphates to inhibit them. Elliott (1945) found that iodoacetic acid inhibited the proteolytic enzymes produced by *Streptococcus pyogenes*, and Gorini (1950) reported that bacterial proteinases were inhibited by citrates. In view of these observations, experiments were made in which iodoacetic acid, phosphates and other substances were tested for their effect on pyocine inhibitors. Finally, a nutrient agar medium was devised containing 10^{-5} molar iodoacetic acid, 0·1 % sodium citrate and 0·1 % dipotassium hydrogen phosphate.

The pyocine-inactivating substances appear to be different from some other pseudomonas proteinases, particularly the one described by Fisher (1960) which is inhibited by thiol groups and stimulated by sodium iodoacetate.

The fact that strains of *Ps. aeruginosa* growing on this medium produce little slime is an additional advantage. Holland (1962) found that the presence of bacterial slime protected otherwise sensitive organisms against the lethal activities of megacine, the bacteriocine produced by *Bacillus megaterium*. Indicator strains which may in fact be sensitive to the bacteriocine may, with abundant slime production, appear to be resistant.

It is considered that the proposed medium will give more consistent and reliable results than simple nutrient media in examining pseudomonas cultures for pyocinogenic activity and is thus of value in investigating the use of pyocine production as the basis for a typing scheme of *Ps. aeruginosa*.

SUMMARY

Certain strains of *Ps. aeruginosa* produce, in addition to pyocines, substances which inhibit pyocine activity. These pyocine inhibitors are probably proteolytic enzymes.

In order to investigate the production of pyocines by various strains of *Ps. aeruginosa*, a nutrient agar medium was devised in which the action of the pyocine-inhibiting substances is suppressed by incorporating 10^{-5}M iodoacetic acid, 0·1 % sodium citrate and 0·1 % dipotassium hydrogen phosphate. This medium also diminished slime production.

Pyocine production is a stable characteristic which is not lost on repeated

sub-culture or prolonged storage, and might form the basis of a typing system for *Ps. aeruginosa*.

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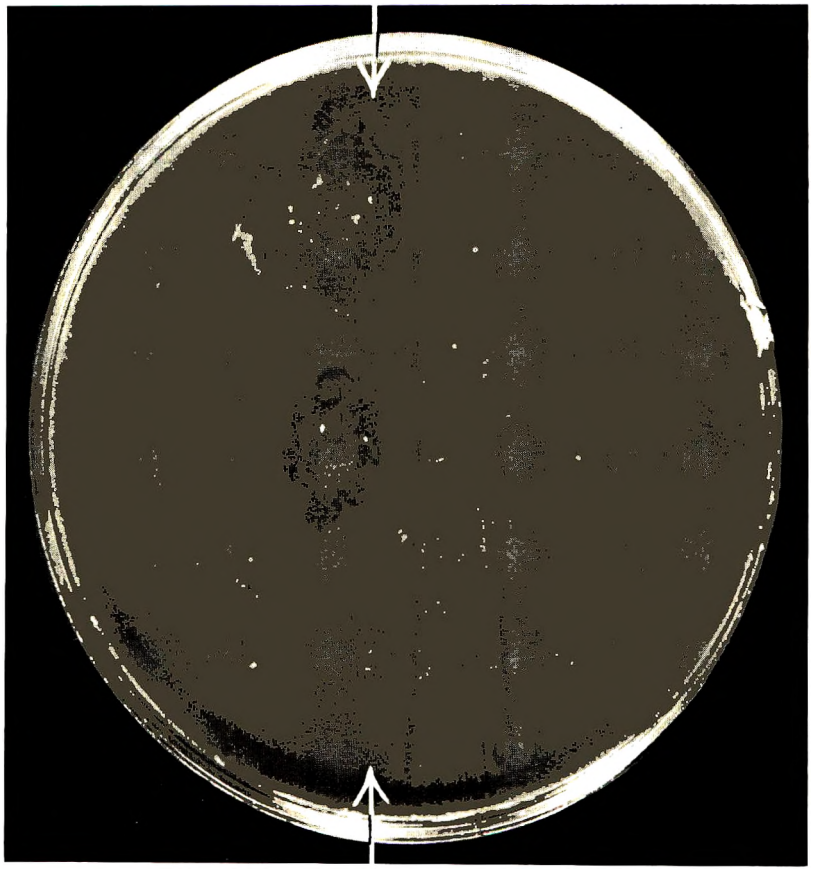

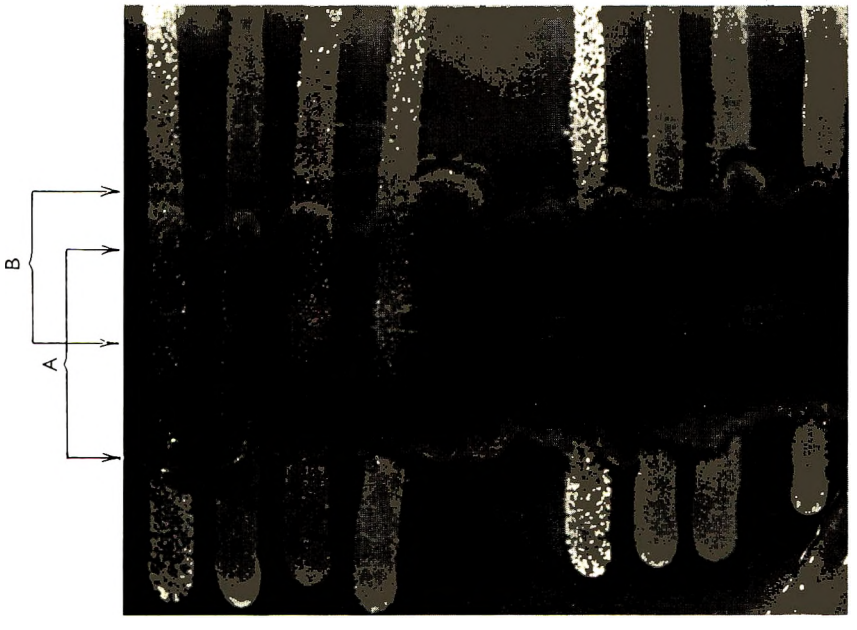

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EXPLANATION OF PLATE 1

Fig. 1. Inactivation of the pyocines of strain M 163 (Streak A) by the non-pyocinogenic strain P 501 (Streak B). In the upper half of the plate, the inactivation of pyocines is shown by growth of the indicator strains on the side of the primary streak where strain P 501 had been grown. Inhibition of indicators occurred on the other side. In the lower half of the plate, where only strain M 163 had been grown, there was inhibition of the indicator strains on both sides.

Fig. 2. Inactivation by the non-pyocinogenic strain W 96 of pyocines produced by strains N.C.T.C. 1999, B 55 and S 178. From left to right: the complete inactivation of pyocines produced by strain N.C.T.C. 1999 is shown by an absent inhibition zone of the indicator M 8. Partial inactivation of pyocines produced by strains B 55 and S 178 is shown by smaller inhibition zones to the side of strain W 96 than to the other side. The arrows point to the line of inoculation of the three pyocinogenic strains.

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The intermittent use of hexachlorophene soap—a controlled trial

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Hexachlorophene has been used as a skin disinfectant during the last 18 years. Various workers have shown that under specified conditions the use of toilet soap containing 2% hexachlorophene reduces the number of bacteria which can be isolated from the skin (Traub, Newhall & Fuller, 1944; Fahlberg, Swan & Seastone, 1948; Price & Bonnet, 1948; Best, Coe, McMurtrey & Henn, 1950; Cade, 1950; Lawrie & Jones, 1952; Hurst, Stuttard & Woodroffe, 1960; Lowbury & Lilly, 1960; Lowbury, Lilly & Bull, 1963). Lowbury & Lilly (1960) showed that the exclusive use of 2% hexachlorophene soap for several days reduced the bacterial flora of the skin of nurses' hands, but its use only during periods of duty was of much less value. Nurses now often live out of the hospital and although it is easy enough to make hexachlorophene soap the only available toilet soap in the hospital, it is impracticable to require all the nursing staff to use hexachlorophene soap when not on duty. However, it is of practical importance to know whether the use of 2% hexachlorophene soap only while on duty does reduce the number of bacteria on the surface of the nurses' hands, and the present controlled experiment was undertaken to settle this point.

There are various techniques for making quantitative estimations of bacteria on the hands and they differ in the depth of the bacterial population sampled. It is probable that only the surface flora of the nurses' hands are likely to be transmitted to patients and therefore are potentially dangerous (Lowbury, Lilly & Bull, 1960). We have therefore used a method by which only surface bacteria are sampled. Other methods such as those which involve washing either the hands or the insides of rubber gloves with sterile solutions from which counts are made (Lowbury & Lilly, 1960; Price, 1938) sample both the superficial and deep bacterial flora. The relevance of the latter is doubtful and these methods are also technically more complicated than the surface sampling method we have used.

METHODS AND DESIGN OF EXPERIMENT

The investigation was conducted in four surgical wards each of thirty beds, in a general hospital of some 400 beds. All the regular nursing staff of the surgical wards were observed during a period of 8 weeks, during which they were required to use either hexachlorophene soap or a control soap while on duty in the hospital. Nurses were told about the experiment, but no nurse knew which periods were the test periods, nor what soaps were being used at any given time during the experiment.

The bacteria on the palms of the hands were counted using the following technique (modified from Lawrie & Jones, 1952). Eight-inch lengths of sterile gauze bandage were embedded in blood agar plates while the medium was still molten. When the medium was cold the disk of agar (approximately 10 cm. in diameter and 0·5 cm. in thickness) was lifted out of the Petri dish and the under side of the disk, which was smooth, was laid on the palm of the hand to be tested. Contact was made for 45 sec. Pressure over the palm was maintained by applying a weight (340 g.) to the upper surface of the blood agar disk. Pressure was distributed uniformly by having a soft rubber inflated bag between the disk and the weight. The surface of the rubber bag under the weight was cleaned by wiping it with a 1:1000 solution of benzalkonium chloride and drying with a sterile swab. This did not interfere with the growth of organisms on the opposite side of the plate.

The blood agar disk was replaced in a Petri dish, contact side uppermost. This was incubated aerobically at 37° C. overnight. Next day a metal template with four square holes, each of 1 cm.^{2} area, was placed on the surface of the medium and the colonies within each of the four squares were counted. Replicate counts of a plate counted by this method showed little variation in the mean for the four squares counted.

The experiment was conducted over 8 consecutive weeks during February and March 1962 in four surgical wards. During the experimental period any nurse on duty in the surgical wards used experimental soap when washing her hands or when washing a patient. While off duty, nurses used whatever soaps or detergents they pleased. Nurses were on duty for 42 hr. a week, usually spread over 6 days, with a maximum of 8 hr. on any one day. While on duty the number of times a nurse washed depended on her duties. For example, nurses handling bedpans washed frequently; those doing dressings scrubbed up before and washed their hands after attending to each patient, and those making beds washed less frequently.

Soap for the experiment was provided by Messrs Bibby and Co. Control soap and soap containing 2% hexachlorophene were each made in two colours, pink and blue, to be distinct from soap used in other wards not in the experiment. Control and hexachlorophene soap of one colour were indistinguishable.

The soaps used were tested *in vitro* before the experiment. Dissolved control or hexachlorophene soap was incorporated in a nutrient agar medium to achieve final concentrations of 1:1000 or 1:10,000 soap. The nutrient agar plates were inoculated with serial dilutions of a strain of *Escherichia coli* or of a strain of *Staphylococcus aureus*. They were then incubated overnight. The growth of *E. coli* was not inhibited by either 1:1000 control soap or 1:1000 hexachlorophene soap (1:50,000 hexachlorophene). The growth of *Staph. aureus* was slightly inhibited on nutrient agar containing 1:1000 control soap and completely inhibited on the medium containing 1:10,000 hexachlorophene soap (1:500,000 hexachlorophene). No differences were observed *in vitro* between pink and blue hexachlorophene soap or between pink and blue control soap.

Soaps were used according to the programme in Table 1. During the 8 weeks the right hand of each nurse was sampled twice a week. The number of hours a nurse had worked since coming on duty was noted. As the concentration of hexachloro-

phene on the surface of the hands was likely to be changing in the first 2 and third 2 weeks the results during these weeks were discarded. Weeks 3 and 4, and weeks 7 and 8 were taken as test periods. The maximum number of estimates for any one nurse during each test period was therefore four. No nurse contributed less than three estimates.

Table 1. *Programme of experiment*

Colour and type of soap by week and by ward

		Equilibration period	First test period	Equilibration period	Second test period
Week of expt.		1, 2	3, 4	5, 6	7, 8
Ward	Type of surgery				
I	Gynaecological and orthopaedic, female	Hexachlorophene (pink)	Hexachlorophene (blue)	Control (pink)	Control (blue)
II	General, male	Control (blue)	Control (pink)	Hexachlorophene (blue)	Hexachlorophene (pink)
III	General, female	Control (pink)	Control (blue)	Hexachlorophene (pink)	Hexachlorophene (blue)
IV	General, genito-urinary and orthopaedic, male	Hexachlorophene (blue)	Hexachlorophene (pink)	Control (blue)	Control (pink)

The changes from pink to blue soap and blue to pink soap were made to allow easy estimation of the amount of soap being used in each ward, to ensure that soaps did not migrate from control to hexachlorophene wards during the experiment, and to check that the colour of soap did not influence the amount of soap used.

RESULTS

Only nurses remaining attached to one ward for at least $3\frac{1}{2}$ weeks of the first or second half of the experiment were included. In spite of excellent co-operation from administrative staff, only four out of ten nurses attached to each ward fulfilled these criteria. Altogether twenty-five nurses were included in the experiment. Of these, seven remained attached to one ward for all 8 weeks of the experiment.

The amount of soap used varied from ward to ward but in any one ward the consumption was remarkably constant. Since each ward contained different types of patient according to the specialities of the consultant surgeons, and since each ward was run on different regimens, the results from all wards for each control or for each hexachlorophene period have been combined, so that each ward contributes equally to the results.

The number of bacteria isolated from the hands of nurses varied widely from day to day, as is shown in the data from two wards in Table 2. The overall variation was not apparently different in the control and hexachlorophene periods. As some nurses were examined only three times and some four times in each test period, the results have been expressed as the mean number of colonies counted in 4 cm.^{2} for each nurse. Therefore for any one test period each of the four wards contributes mean counts from each of four nurses.

Table 3 shows the mean values for each nurse for all four wards in the experiment. The difference between mean counts obtained during the hexachlorophene period and during the control period is not significant ($P > 0{\cdot}4$).

Of the seven nurses who did not move from one ward to another during all

Table 2. *Number of colonies in 4 cm.² from fifty-eight contact samples from palms of nurses' hands (two wards only)*

Soap used	Ward	Nurse	Days after exposure to soap: 16	20	23	27
Control	III	A	8	6	8	24
		B	23	5	137	11
		C	16	—	418	3
		D	215	3	2	5
Control	IV	I	2	1	0	16
		K	45	61	35	5
		L	7	12	18	—
		M	3	2	185	25
2% hexachlorophene soap	III	E	6	1	14	83
		C	19	30	8	105
		D	509	19	11	9
		F	4	206	4	28
2% hexachlorophene soap	IV	G	66	31	10	0
		H	22	30	1	20
		I	0	3	5	2
		J	9	421	51	—

Table 3. *Mean of counts in 4 cm.² for each nurse from all four wards during control period and during hexachlorophene period*

	Control period			Hexachlorophene period		
Ward	Soap colour	Nurse	Mean count	Soap colour	Nurse	Mean count
I	Blue	T	2	Blue	T	31
		X	18		U	31
		Y	46		V	21
		W	56		W	9
II	Pink	N	13	Pink	R	67
		O	9		O	41
		P	30		S	24
		Q	22		Q	42
III	Blue	A	12	Blue	E	26
		B	44		F	61
		C	146		C	41
		D	56		D	137
IV	Pink	I	5	Pink	I	3
		K	37		G	27
		L	12		H	18
		M	54		J	160
Mean = 35·13 ± S.E. 9·07				Mean = 46·18 ± S.E. 11·92		

8 weeks of the experiment, the mean during the control period was 42·3 ± S.E. 19·3 colonies in 4 cm.^{2} which did not differ from the mean value of 43·4 ± S.E. 16·7 colonies per 4 cm.^{2} obtained during the hexachlorophene period.

It was thought that the length of time on duty before the hands were sampled might affect the number of organisms on the hands. Table 4 shows the mean of colonies per 4 cm.^{2} analysed by time on duty. In fact mean counts obtained were higher after duty in the hexachlorophene periods than during the control periods, but the differences were not significant. Similarly, and equally without significance, there were less organisms on the hands of nurses arriving on duty in the hexachlorophene periods than on the hands of nurses arriving on duty during the control period.

Table 4. *Mean number of colonies and standard error in 4 cm.^{2} from all nurses' hands after being on duty for varying periods*

(Number of observations in brackets)

	Hours on duty						
	0	1	2	3	4	5	6
During control period	61·4 ± 16·56 (16)	13·7 (1)	17·5 (2)	3 (3)	36·7 ± 27·39 (15)	15·1 ± 5·08 (18)	14 (3)
During hexachlorophene period	28·5 ± 8·63 (13)	10 (3)	96·3 (6)	Nil	57·2 ± 6·64 (21)	36·8 ± 13·76 (16)	15 (2)
Difference between means. Hexachlorophene minus control	−32·9	—	—	—	+20·5	+21·7	—
Probability of difference being due to chance	$0·3 < P < 0·4$	—	—	—	$0·5 < P < 0·6$	$0·1 < P < 0·2$	—

Table 5. *Number of nurses carrying coagulase positive staphylococci in wards using hexachlorophene soap and control soap*

	Nurses carrying coagulase positive organisms	Nurses not carrying coagulase positive organisms	Total
Control soap	5	9	14
Hexachlorophene soap	9	6	15

For the difference between these proportions: $\chi^2 = 1·71$ $0·2 > P > 0·1$

At the beginning of the experiment a number of staphylococci which had been isolated from the hands of nurses in hexachlorophene wards and in control wards were tested for coagulase. The few results shown in Table 5 indicate no significant difference in the incidence of coagulase positive staphylococci on nurses' hands between the control and hexachlorophene wards. Apart from this, no attempt was made to identify the organisms recovered from nurses' hands during the experiments.

DISCUSSION

In this experiment the effects of 2 % hexachlorophene soap on the bacteria on the surface of nurses' hands have been studied by contact sampling and it was found that this soap did not reduce the number of viable bacteria on the skin. Both Best *et al.* (1950) and Lowbury & Lilly (1960) studied bacteria on the hands of nurses. Although their sampling methods differed, both sets of workers found that regular and exclusive use of 2 % hexachlorophene soap resulted in a reduction of viable bacteria on the hands. However, Lowbury & Lilly (1960), in their broad study which is not directly comparable to the limited controlled experiment reported here, showed that the intermittent use of hexachlorophene soap by nurses was of doubtful value in reducing bacteria on the hands.

Fahlberg *et al.* (1948) have shown that an ether soluble substance which inhibited the growth of *Staph. albus* was present on the skin of subjects who had used hexachlorophene soap for several days, and the concentration of this substance decreased during 72 hr. after hexachlorophene soap had been last used. It seems probable that the substance which accumulated was hexachlorophene and that the effectiveness of hexachlorophene soap depends on the accumulation of hexachlorophene on the skin.

In the present investigation nurses used hexachlorophene soap only while at work. Any washing with other soaps or detergents while not at work probably removed any hexachlorophene which had accumulated during work, so that no effective antibacterial concentration of hexachlorophene was ever reached.

This work shows that the intermittent use of hexachlorophene soap has no effect in reducing the number of superficial bacteria on the hands.

SUMMARY

The number of bacteria on the hands of nurses using 2 % hexachlorophene soap intermittently was compared with the numbers of bacteria on the hands of nurses using ordinary soap. No significant differences were observed.

This study would not have been possible without the whole-hearted co-operation of the Matron, Miss M. Schurr, the Deputy Matron, Miss G. Davies and the Sisters and Nursing Staff of Fulham Hospital.

We are also indebted to Mr T. Ridgewell, Mr T. F. Fletcher and Miss Julia Fisher for technical assistance, and to Messrs Bibby and Co. for their generous gift of soap.

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A slowly sedimenting infectious component of Rift Valley fever virus

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INTRODUCTION

It is now known that many viruses contain infective particles differing in sedimentation constant. Type II poliomyelitis, for example, consists of a mixture of particles having sedimentation constants of $S_{20} = 170$ and $S_{20} = 100$ (Selzer & Polson, 1954). Of special interest are viruses containing infectious components with exceptionally low sedimentation constants. Hampton (1958) has mentioned the existence of such material in West Nile virus. Kipps, Turner & Polson (1961) estimated the sedimentation constant of the slowly sedimenting fraction of CBO virus as 20*S* and Gessler, Bender & Parkinson (1956) working with Rous sarcoma virus purified with fluorocarbon found high tumour producing activity in the supernatant fluid after centrifugation at 144,000 *g* for 20 hr.

In this laboratory it has been observed that on centrifugation of Rift Valley fever virus (RVF), under conditions which sedimented yellow fever (Polson, 1954), African horsesickness (Polson & Madsen, 1954), MEF_1 polio (Polson & Selzer, 1952) and Semliki Forest viruses (Cheng, 1961) from the supernatant fluid, the virus activity could be reduced rapidly by about 99 %. The remaining 1 % of virus could be reduced only slightly by further repeated centrifugations. This apparent inhomogeneity of RVF virus has now been further investigated.

MATERIALS AND METHODS

Viruses

Rift Valley fever

Pantropic Rift Valley fever virus obtained from the Director, Department of Veterinary Research, Onderstepoort, Pretoria, was maintained by intraperitoneal mouse passage. Defibrinated infected blood from adult white mice or a 20 % suspension of liver and spleen from mice 15 to 19 days old were used as sources of virus.

Polio

The MEF_1 strain of type II poliovirus (Selzer, Sacks & van den Ende, 1952) in its 225th passage in mice as a 10 % suspension of 4- to 5-day-old infected unweaned-mouse brains was used.

RVF antiserum

This was prepared in adult mice by eight intraperitoneal injections of a 10 % suspension of infected mouse liver or spleen. The first two injections contained 0·016 % formalin.

Deoxyribonucleic acid and nucleases

Calf thymus DNA, crystalline deoxyribonuclease and ribonuclease were obtained from Seravac Laboratories, Cape Town.

Virus titration

The diluent used for virus suspensions and titrations was a mixture of 5 % (v/v) rabbit serum with 0·85 % (w/v) saline containing 200 units of penicillin and 0·2 mg. of streptomycin per ml.

RVF

Tenfold serial dilutions were made and each was tested in six mice, 3–5 weeks old. Each animal received 0·2 ml. intraperitoneally. Titres are expressed as the negative $\log_{10}$ LD 50 per 0·2 ml., calculated by the method of Reed & Muench (1938). Infectivity units are the antilog values of the negative $\log_{10}$ LD 50.

MEF_1

Each dilution was titrated in a litter of eight unweaned mice, 4–5 days old, 0·02 ml. being injected intracerebrally. In the caesium chloride density gradient centrifugation experiments, 0·03 ml. of each fraction was injected intracerebrally into six 3- to 4-week-old mice. Titres are the negative $\log_{10}$ LD 50 per 0·02 or 0·03 ml.

Complement fixation

Complement-fixation tests were performed as described by Casals & Olitsky (1950). Tenfold or doubling dilutions of antigen were tested against the antiserum diluted 1/10 and 1/100. The diluent (pH 7·2) contained Mg and Ca (Mayer, Osler, Bier & Heidelberger, 1946). Complement was titrated by the method of Casals, Olitsky & Anslow (1951) and 2 units were used in the test. The antiserum was inactivated at 56° C. for 30 min.

The complement-fixing titre or one CF unit is equal to the reciprocal of the highest dilution of antigen giving 50 % fixation.

Sedimentation constants

These were measured in the model LH Spinco centrifuge by either of the following methods.

Method 1, using the no. 40 rotor was described by Polson & Linder (1953).

The average rotor velocity was determined by the method of Polson & Madsen (1954). *Jasus lalandii* haemocyanin (S_{20} = 16) added as a sedimentation reference formed a density gradient which stabilizes the virus boundary. MEF_1 poliovirus was ultracentrifuged simultaneously in another tube as an additional control.

Method II, using the SW 39 rotor was described as method B by Polson & van Regenmortel (1961).

In view of the prolonged centrifugation, the virus suspension was layered over 20 % (w/v) bovine plasma albumin (fraction V) in effective column lengths which ranged between 3 and 8 mm. instead of over 40 % (w/v) sucrose.

Centrifugation in density gradients

Densities were determined in a preformed gradient of caesium chloride (Analar, The British Drug Houses Ltd.), the virus suspension being introduced into the gradient close to its isodensity level (Polson & Levitt, 1963).

Estimation of haemocyanin

Samples containing haemocyanin were assayed by immuno-diffusion as described by Polson & van Regenmortel (1961).

Ultrafiltration

The gradacol membranes used were prepared and calibrated in our laboratory.

Gel diffusion-filtration

The column 45 × 2 cm. containing granulated 7 % Difco agar gel (60 mesh) was described by Polson (1961*a*). The displacement medium (pH 7·0) contained 0·067 M phosphate and 0·073 M-NaCl. Fractions were collected in an automatic collector (Polson, 1961*b*).

The following were used as reference proteins:

(*a*) Haemocyanin of *Burnupena cincta*, S_{20} 89 and 92, molecular weight 6,600,000 and diffusion coefficient $1{\cdot}24 \times 10^{-7}$ cm.²/sec. (Polson & Deeks, 1960).

(*b*) Haemocyanin of *Jasus lalandii*, S_{20} 16, molecular weight 490,000 and diffusion coefficient $3{\cdot}4 \times 10^{-7}$ cm.²/sec. (Joubert, 1954; Polson, 1956).

(*c*) Erythrocruorin of *Arenicola loveni*, S_{20} 60, molecular weight 3,200,000 (Svedberg & Pedersen, 1940).

(*d*) Rabbit or mouse haemoglobin, S_{20} 4·36, molecular weight 68,000 and diffusion coefficient $6{\cdot}5 \times 10^{-7}$ cm.²/sec. (Svedberg & Pedersen, 1940; Polson, 1961*a*).

The opacity near 253 mμ of the column effluent was recorded by an LKB Produkter Uvicord coupled to an Esterline–Angus recording milliammeter.

EXPERIMENTAL AND RESULTS

Preparation of S_{low} *particle material*

To remove all rapidly sedimenting virus, a 20 % suspension of RVF-infected liver and spleen in serum-saline, or infected serum, was centrifuged in the no. 40 rotor four times successively at 33,000 r.p.m. for 60 min. under liquid paraffin. This was added to bind at the interface any lipoids or lipoproteins which might have carried virus towards the surface. After each centrifugation, the supernatant fluid was carefully removed by a pipette held initially half-way between the

liquid paraffin layer and the bottom of the tube and lowered only enough to remove fluid to a level 1 cm. above the bottom of the tube. The remaining fluid was discarded. The pooled supernatants were centrifuged in clean tubes. The final supernatant fluid was regarded as the S_{low} particle material.

After each centrifugation a sample of the supernatant fluid was titrated in mice for infectivity. Supernatants from liver and spleen which contained more soluble antigen than serum were also titrated for complement fixation. The results are given in Fig. 1. The upper curves show the initial infectivity titres and the changes

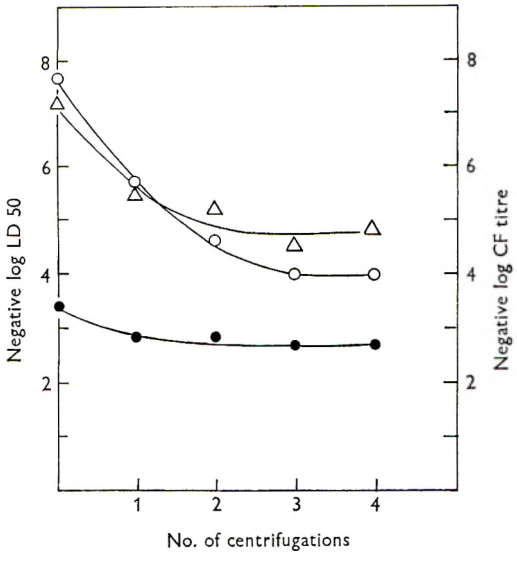

Fig. 1. The changes in infectivity and CF titre of the supernatant fluid during four 1 hr. periods of centrifuging RVF at 33,000 r.p.m. in the no. 40 rotor. The two upper curves are from different experiments. Sedimentation of RVF CF antigen (full circles) and infective virus in two separate experiments (triangles and open circles) on four successive periods of centrifugation at 33,000 r.p.m. for one hour.

in titre of the supernatant fluid after one-hour periods of centrifugation in two experiments. The lower curve shows the initial CF titre and the change in CF titre during successive centrifugations in one experiment.

During the first centrifugation of 1 hr. at 33,000 r.p.m., there was a 100-fold reduction of infective virus in the supernatant fluid. After another slight drop in titre during the second centrifugation, the curve reached a plateau in the region of a negative log LD 50 4·5. The CF antigen showed less reduction in titre during successive centrifugations.

Method I *Determination of sedimentation constants*

Mouse serum infected with RVF was centrifuged twice under liquid paraffin at 30,000 r.p.m. for 60 min. and 9·0 ml. of the supernatant was mixed with 2·0 ml. *Jasus lalandii* haemocyanin. The mixture was placed in a Spinco rotor no. 40 centrifuge tube marked off into ten 0·5 cm. divisions, starting 1·5 cm. from the

top, the eleventh mark being 1 cm. from the bottom of the tube. The tube was placed in the rotor, which was surrounded by ice until the temperature of the water-balance tube was 2·5° C. The rotor was then spun at 33,000 r.p.m. for 90 min. The rotor velocity was noted at intervals, during acceleration, during the main centrifugation period and during deceleration. Eleven samples were taken from the tube. Each sample was titrated for infectivity in mice and assayed for haemocyanin by the gel precipitin technique.

In this experiment (Fig. 2) there was only slight sedimentation of the haemocyanin and the presence of a faster sedimenting virus component was indicated

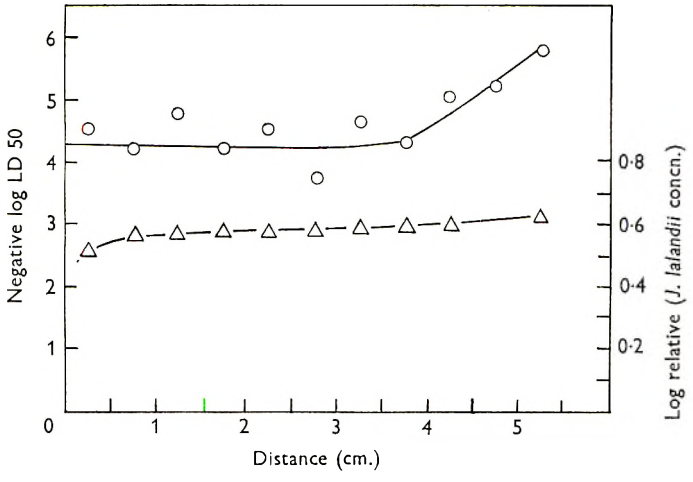

Fig. 2. Sedimentation at 33,000 r.p.m. for 90 min. in the presence of *J. lalandii* haemocyanin, of a suspension of RVF virus which had been previously centrifuged twice at 30,000 r.p.m. for 60 min. The circles represent infectivity titres of successive 1 cm. samples from the tube. The triangles represent the relative concentrations of *J. lalandii* haemocyanin.

by the steep slope of the curve beyond the 4 cm. level in the tube. In succeeding experiments the faster sedimenting virus was eliminated.

In a second experiment a 20 % suspension of liver and spleen infected with RVF was centrifuged for 60 min. at 30,000 r.p.m. The supernatant fluid was removed and submitted to the same treatment. The second supernatant fluid was mixed with *J. lalandii* and centrifuged 90 min. at 33,000 r.p.m. The upper 4 cm. layer of fluid was then carefully removed for the particle size determination. A 10 % suspension of MEF_1 infected mouse brains was treated in the same way to act as a control.

Three centrifuge tubes were marked as described above. The first and second tubes contained RVF and MEF_1—*J. lalandii* haemocyanin mixtures, respectively, the third tube contained 9·0 ml. serum-saline and 2·0 ml. haemocyanin and the fourth, a water balance, was used for temperature measurement. The tubes were centrifuged at 33,000 r.p.m. for 5 hr. and their contents divided into fractions and assayed with the results shown in Fig. 3B. No titres of MEF_1 could be included because it was subsequently found that an MEF_1 suspension of negative log

LD 50 = 5 contained no detectable infective virus after the three preliminary centrifugations to which the RVF had been subjected.

The third experiment resembled the second except that the MEF_1 virus suspension was merely clarified prior to the 5 hr. centrifugation and not subjected to the preliminary three cycles of centrifugation. The results are shown in Fig. 3A.

A fourth experiment was performed on RVF virus alone (Fig. 3C).

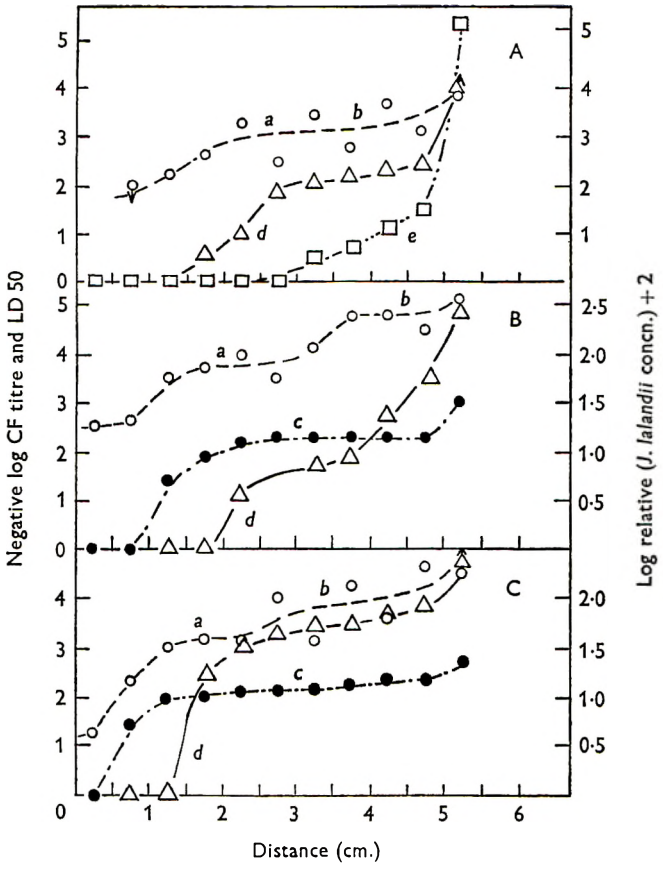

Fig. 3. Sedimentation of S_{low} RVF and MEF_1 poliovirus during 5 hr. at 33,000 r.p.m. in the presence of *J. lalandii* haemocyanin. Diagrams A, B and C refer to different experiments. For the preparation of the S_{low} RVF see text. The points refer to successive 0·5 cm. layers. Curves *a*, *b*—infectivity; curve *c*—complement fixation; curve *d*—relative haemocyanin concentration; curve *e*—infectivity titre of poliovirus.

In Fig. 3, curves (*ab*) show the S_{low} virus content of the samples taken at different levels in the centrifuge tube. Curves (*c*) represent the complement-fixing titres expressed as negative log values. Curves (*d*) indicate the relative haemocyanin concentration expressed logarithmically. Curve (*e*) in Fig. 3A represents the negative log LD 50 values of MEF_1 virus at various levels in the tube.

The sedimentation constants given in Table 1 were calculated from these curves by the formula

$$S_{20} = \frac{2H \sin \alpha}{(2x_1 + H \sin \alpha)\omega^2(t_2 - t_1)} \frac{\eta_T}{\eta_{20}},$$

where x_1 = distance of the initial boundary from the axis of rotation, α is the angle of inclination of the tube (26°), ω the angular velocity of the rotor, $(t_2 - t_1)$ the effective time of centrifugation (300 min. in these experiments) and η_T/η_{20} the ratio of the viscosity of the solution at the temperature of centrifugation to that of water at 20° C. The position of the sedimenting virus boundary H in the tube, which corresponds to the 50 % concentration point in the Svedberg light-absorption technique, is determined from the position of a line drawn through a point representing a titre T, where $T = \log \frac{1}{2}(\text{antilog } T_2 - \text{antilog } T_1)$ and T_2 is the virus titre below, and T_1 that of the titre above the boundary zone respectively (Polson & Madsen, 1954).

The infectivity titres obtained with the S_{low} virus samples after ultracentrifugation suggested the presence of more than one component. In Fig. 3B and C the sedimentation diagrams of the S_{low} virus were drawn as two-step curves, but owing to the distribution of points in Fig. 3A, the diagram could only be drawn as a single curve. Sedimentation constants were calculated from the two plateaux *a* and *b* in Fig. 3B and C. The component giving rise to the portion (*a*) of the curves has a sedimentation constant of about 7, while that giving rise to (*b*) appears to have a value between 15 and 19. The CF antigen (curve *c*) has an estimated sedimentation constant between 7 and 8·6.

Variation in the position of the curves in Fig. 3B and C is due to the difference in temperature at which the two ultracentrifugation experiments were conducted (see Table 1).

The *J. lalandii* haemocyanin used for these experiments contained dissociation products which form during storage and give rise to sedimentation curves (*d*) unsuitable for accurate estimate of sedimentation constants.

Table 1. *Calculation of sedimentation constants of RVF* S_{low} *material and complement-fixing antigen*

X_1 = 5·13 cm.; $t_2 - t_1$ = 300 min.; $\sin_\alpha$ = 0·4384

Material	Curve	Fig.	Effective rotor velocity (r.p.m.)	Rotor equilibrium temperature (° C.)	H (cm.)	η_T/η_{20}	S_{20}
RVF S_{low}	*a*	3B	33,230	10·5	1·5	1·312	7·2
RVF S_{low}	*b*				3·5	1·312	15·6
RVF CF antigen	*c*				1·8	1·312	8·6
RVF S_{low}	*a*	3C	33,020	2·25	1·1	1·691	7·1
RVF S_{low}	*b*				3·2	1·691	18·9
RVF CF antigen	*c*				1·1	1·691	7·1

The haemocyanin, by forming a density gradient in the tube, was valuable as a hindrance to convection and the position of its boundary proved further evidence of the smallness of the sedimentation constants of the slower sedimenting S_{low} (*a*) particle and CF antigen.

The MEF_1 virus (Fig. 3A, curve *e*) had an initial infectivity titre similar to that of the S_{low} material, but after centrifugation for 300 min. at 33,000 r.p.m. had

sedimented almost completely into a pellet and only traces were detected near the bottom of the tube. In contrast to this, the RVF S_{low} fraction maintained a negative log LD 50 of about 3 below a level 2 cm. from the top of the tube.

Method II

A suspension of RVF S_{low} virus prepared from infected mouse serum was layered over 20% (w/v) albumin in effective column lengths of 3–8 mm. The albumin diffused to form a steep gradient immediately below the effective virus column and prevented the virus from moving back into the effective column by convection. The tubes were centrifuged at 33,000 r.p.m. for 5 hr. at or near 0° C. After centrifugation, the whole of the fluid above the albumin gradient was removed and titrated in mice. The sedimentation constant was calculated from the equation

$$S_{20} = \frac{3{\cdot}50 \log X}{N^2 t} \frac{\eta_T}{\eta_{20}},$$

where η_T and η_{20} are the viscosities of the dispersion medium at the temperature of centrifugation and of water at 20° C., respectively, N the rotor velocity in rev./min., and t the time of centrifugation in minutes,

$$X = \frac{x+l}{x+l(C_t/C_0)},$$

in which x is the distance from the upper meniscus to the centre of rotation, l is the effective column length (i.e. the distance between the upper meniscus and the upper limit of the albumin gradient) and C_t/C_0 the ratio of the final to the initial concentration of virus in the effective column.

Results by this method (Table 2) indicated a value of about 4S for the sedimentation constant of the S_{low} virus fraction.

Table 2. *The sedimentation constant of RVF* S_{low} *virus calculated by centrifugation in columns of different length*

Negative $\log_{10}$ LD 50 of original S_{low} fraction (C_0) = 3·50, N = 33,000 r.p.m., η_{20} = 0·010, η_T = 0·018, t = 300 min., x = 5·85 cm.

Effective column length (cm.)	Negative $\log_{10}$ LD 50 (C_t)	T (° C.)	C_t/C_0	S_{20}
0·3	2·60	0	0·13	3·6
0·4	2·75	0	0·18	4·5
0·5	3·15	0	0·45	3·8
0·6	3·25	0	0·56	—
0·7	3·50	1	1·00	—
0·8	3·25	1	0·56	—
			Average	4·0

Centrifugation in a density gradient

Using whole infected mouse serum containing both the S_{low} fraction and the sedimentable portion, Polson & Levitt (1963) found a density of 1·23 g./cm.^{3} for the virus. The densities of the S_{low} and sedimentable virus have now been separately determined.

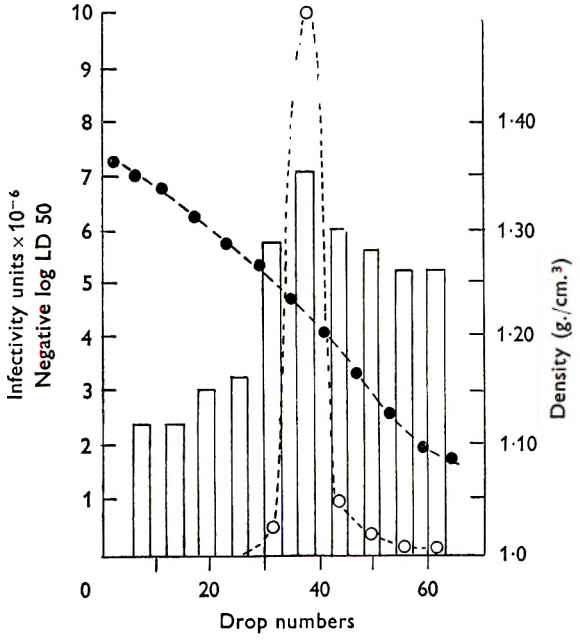

Fig. 4. Density gradient centrifugation of RVF virus (sedimentation constant 452 *S*). The virus was introduced near the centre of a preformed CsCl gradient. Histogram, negative log LD 50 values. Open circles, infectivity units (antilog values). Full circles, distribution of density after centrifugation.

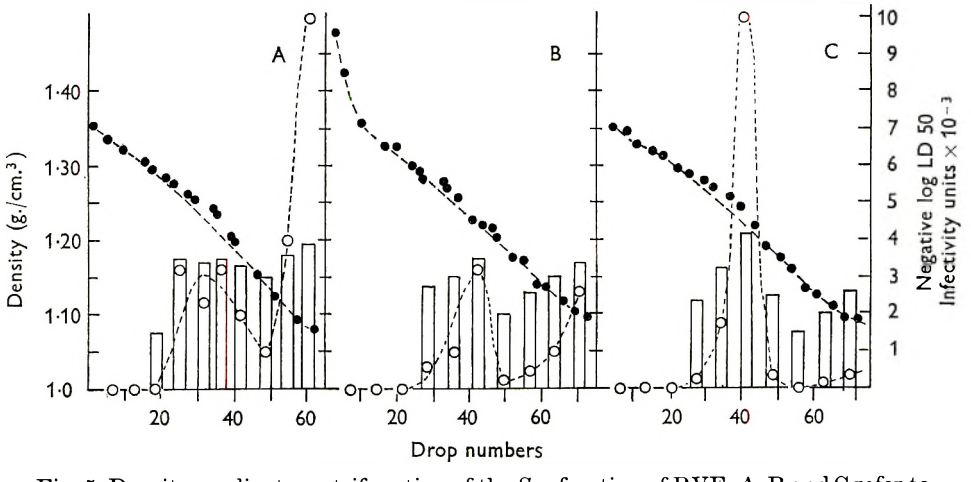

Fig. 5. Density gradient, centrifugation of the S_{low} fraction of RVF. A, B and C refer to separate experiments in which the virus was introduced at the centre of a preformed gradient. Histograms, negative log LD 50 values. Open circles, infectivity units (antilog values). Full circles, distribution of density after centrifugation.

Mouse serum (10 ml.) infected with RVF virus was centrifuged at 30,000 r.p.m. for 90 min. to sediment the larger virus particles into a pellet. The pellet was resuspended in serum-saline (1 ml.) and the suspension was mixed with enough

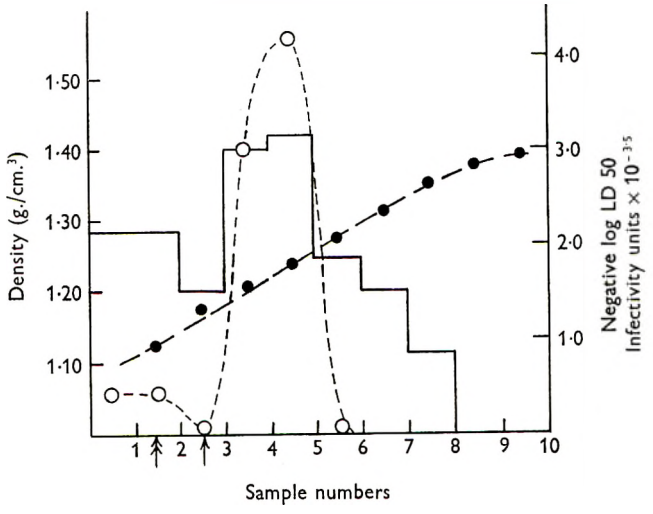

Fig. 6. Density gradient centrifugation of the S_{low} fraction of RVF in the presence of haemoglobin. In this experiment the virus was introduced at a density level of 1·09 g./cm.³ and the tube was sampled from the top. Histogram, negative log LD 50 values. Open circles, antilog values (infectivity units). Full circles, density gradient after centrifugation. Double arrows, point of introduction of virus and haemoglobin. Single arrow, position of haemoglobin after centrifugation.

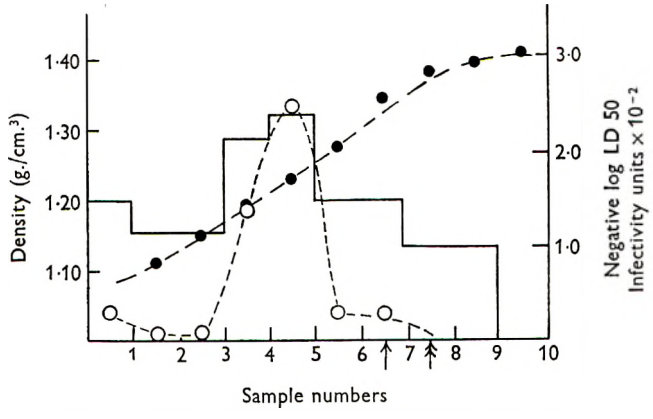

Fig. 7. Density gradient centrifugation of the S_{low} fraction of RVF virus in the presence of haemoglobin. In this experiment the virus sample was introduced at a density level of 1·36 g./cm.³. Samples taken from the top. Histogram, negative log LD 50 values. Open circles, antilog values (infectivity units). Full circles, density gradient after centrifugation. Double arrows, point of introduction of virus and haemoglobin. Single arrow, position of haemoglobin after centrifugation.

caesium chloride solution to bring the density of the mixture to 1·22 g./cm.³ and introduced in the centre of a CsCl density gradient. The tube was centrifuged for 300 min. at 33,000 r.p.m. Samples were taken and examined as described by

Polson & Levitt (1963). The results (Fig. 4) indicated a density of 1·23 g./cm.^{3} for the rapidly sedimenting particles of which the virus is mainly composed.

Fig. 5A, B and C shows the results of three experiments made in the same way but with the S_{low} fraction. There appears to be a main component of density 1·23 g./cm.^{3} and a variable amount of components of lower density.

In an attempt to obtain more definite results, the S_{low} virus sample was introduced into gradients on either side of the expected density level. On this occasion the centrifuge tubes were sampled from the top to ensure that the fractions taken

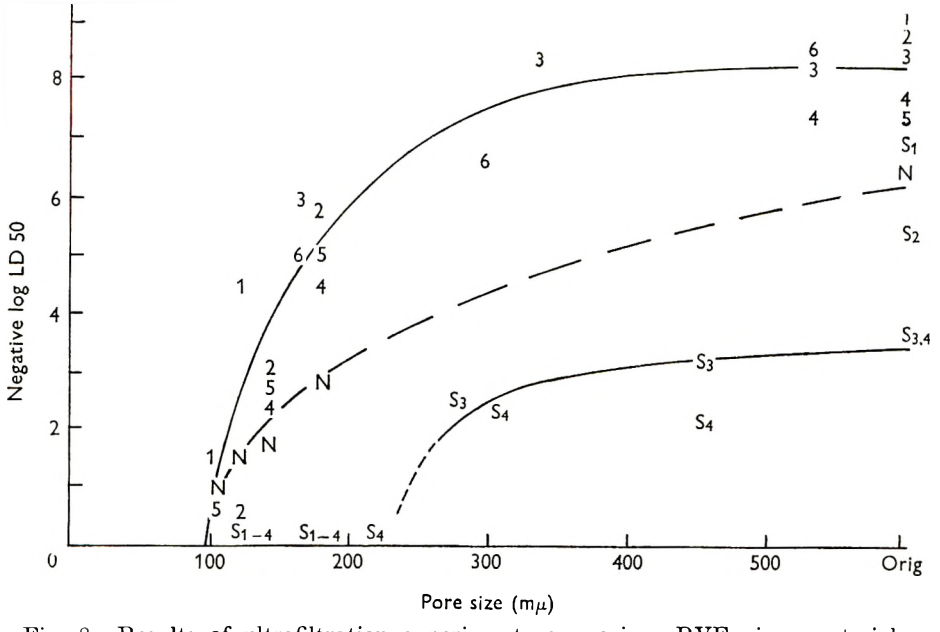

Fig. 8. Results of ultrafiltration experiments on various RVF virus materials. Ordinates are titres in filtrates expressed as neg. log LD 50 values and abscissae membrane pore sizes in mμ. The *S*'s indicate the titre of the S_{low} material and the *N*'s that of the neurotropic strain of RVF. The *S*'s on the base line at log LD 50 = 0 mean that no virus could be detected in the filtrates. The numbers indicate the titres of the filtrates of the 'whole' pantropic virus in different experiments. The titres of the stock virus used are given on the ordinate on the right side of the diagram.

at and near the meniscus were not contaminated with virus from other parts of the gradient. A RVF S_{low} preparation was mixed with CsCl to form two virus suspensions having densities 1·09 and 1·36 g./cm.^{3}. These were introduced into two separate density gradient tubes in place of samples 2 and 8, respectively, and centrifuged in the usual manner. Ten samples were taken from the top of the tube using separate pipettes for each. The results of this experiment are shown in Figs. 6 and 7.

In Fig. 6 it may be seen that most of the infective virus moved down from a density region of 1·09 where it was introduced to a region of density 1·23 g./cm.^{3}. Similarly Fig. 7 shows that most of the virus introduced at a density of 1·36 moved up to a density level of 1·23 g./cm.^{3}. However, it should be noted that in each case a small fraction of virus rose to the surface of the CsCl gradient.

Ultrafiltration

Ultrafiltration experiments were performed on defibrinated RVF infected serum containing the *S* 450 particles as well as on the infected serum freed of the *S* 450 particles. In addition, ultrafiltration experiments were done on the neurotropic strain of the virus, which had received 105 intracerebral passages in mouse brain. In Fig. 8 is given a composite diagram of the results. It will be noticed that the 'whole' virus in the infected serum and the neurotropic strain showed the conventional ultrafiltration curves for viruses. The filtration end-point for both strains was approximately 100 mμ. In contrast to this, the S_{low} material showed a filtration end-point between 219 and 278 mμ, a range of pore sizes through which the 'whole' virus filtered quite readily.

Gel diffusion-filtration

Further information about the particles of the S_{low} fraction of RVF virus was obtained from diffusion-filtration experiments with agar gel.

Mouse serum infected with RVF was centrifuged twice under liquid paraffin at 30,000 r.p.m. for 60 min. The centrifuged serum (1 ml.) was mixed with *Burnupena cincta* haemocyanin (1 ml.) and rabbit haemoglobin (0·1 ml.) and placed on a column (45 × 2 cm.) of granulated agar gel which had passed a 60 mesh sieve. The fraction collector was started when 35 ml. of buffer had flowed from the column and forty-eight fractions of about 1·8 ml. were collected. Every fourth fraction was titrated in mice and the numbers of the fractions containing the reference proteins were noted (Fig. 9A).

In a similar experiment with 2·0 ml. of infected serum which had been pretreated in the same manner, rabbit haemoglobin (0·1 ml.) and *Arenicola loveni* erythrocruorin (0·3 ml.) were added as reference proteins (Fig. 9B).

For comparison an exactly similar experiment was made with MEF_1 poliovirus purified by two cycles of low- and high-speed centrifugation. The reference proteins were rabbit haemoglobin and *A. loveni* erythrocruorin (Fig. 9C). The results showed that whereas poliovirus (S_{20} = 156) emerged from the column in a relatively narrow zone very slightly in advance of the *A. loveni* erythrocruorin (S_{20} = 60), the RVF virus began to come out before the erythrocruorin and even before the *Burnupena cincta* pigment (*S* = 89 – 92). RVF virus did not leave the column in as sharp a zone as the MEF_1, but the possibility that the RVF used for these experiments contained some *S* 450 particles cannot be excluded.

RVF S_{low} material which had been centrifuged four times was also tested by gel diffusion-filtration. First, 2·5 ml. of the S_{low} virus suspension derived from liver and spleen, containing haemoglobin and other liver pigments but no added proteins, was applied to the column and fractions collected as before. Every fifth fraction was titrated in mice and tested by complement fixation. When all the protein components had emerged, 1·5 ml. of the S_{low} virus suspension containing 0·5 ml. of the *B. cincta* and 0·5 ml. of the *Jasus lalandii* haemocyanins were run through at the same flow rate and an ultra-violet opacity record of the emerging protein zones were made. The speed of the recording milliammeter chart and that of the fraction

collector were known. By correlating the haemoglobin peak on the chart with the numbers of the fractions in which haemoglobin was visible, it was possible to locate the position of peak virus infectivity and peak CF activity in relation to the reference proteins. In Fig. 10, diagram B shows the ultra-violet opacity of each

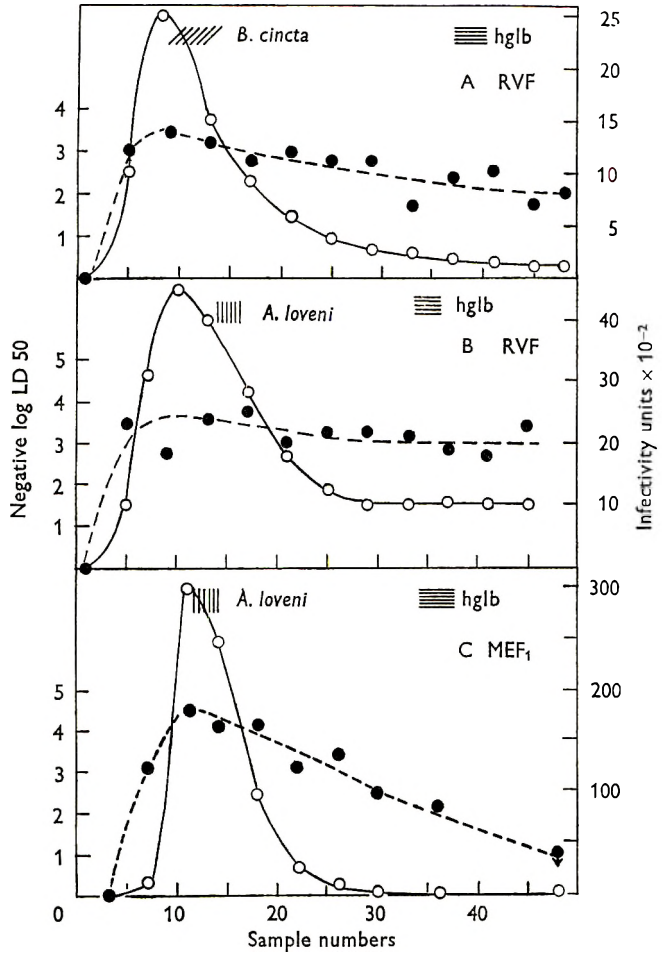

Fig. 9. Diffusion-filtration of, A and B, RVF virus (previously centrifuged twice at 30,000 r.p.m. for 60 min.) and, C, MEF_1 poliovirus in a column of granulated 7% agar gel in the presence of haemoglobin (hglb) and the erythrocruorin of *A. loveni* and haemocyanin of *B. cincta*. Full circles, negative log LD 50 values. Open circles, antilog values (infectivity units). The position of the pigments in the filtrate are shown by shaded areas.

fraction, diagram C the relative virus concentration in every fifth fraction, and diagram D the CF activity of the fraction assayed for virus. The marker pigments responsible for the ultra-violet opacity peaks are indicated in diagram B. Diagram A is the 'elution' curve of *J. lalandii* haemocyanin alone. In this experiment also the virus began to emerge before the *B. cincta* pigment. Most of the CF antigen came out much later than the major part of the virus.

The gel diffusion-filtration behaviour of the denser component of the S_{low} fraction was next investigated.

The S_{low} fraction from mouse serum was centrifuged in a CsCl gradient as previously described and the fraction containing the virus component of density 1·23 g./cm.^{3} was dialysed to remove CsCl and placed on the agar column. In this experiment rabbit serum (5 %, v/v) was added to the displacement medium to stabilize virus infectivity and the column was equilibrated with this mixture before the virus was applied. Every third fraction was titrated in mice.

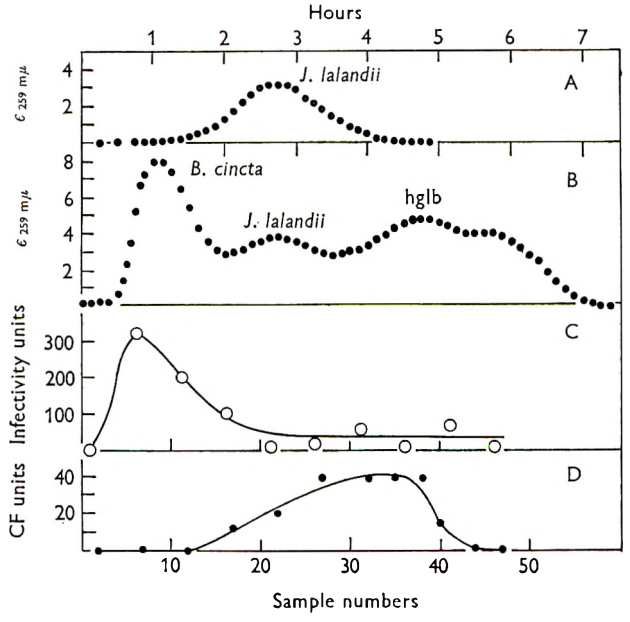

Fig. 10. Gel diffusion-filtration of: A, *J. lalandii* haemocyanin. B, *B. cincta*, *J. lalandii* haemocyanins and haemoglobin (hglb) in a suspension of mouse liver and spleen. C, RVF S_{low} virus fraction. D, RVF complement-fixing antigen present in the S_{low} virus preparation.

The infectivity curve resembled closely that of the whole S_{low} fraction (Fig. 10C), indicating that the denser S_{low} particle diffuses less readily into agar than *B. cincta* haemocyanin and therefore presumably has a lower diffusion constant (Polson, 1961*a*).

In view of the possibility that S_{low} RVF may be filamentous, the gel-diffusion filtration behaviour of deoxyribonucleic acid known to consist of filamentous molecules was examined on a similar agar column (60 × 2 cm.) consisting of 100 mesh 7 % Ionagar no. 2 (Oxoid).

Calf thymus DNA of high molecular weight (4 mg.) in 2 ml. of the buffered saline medium was applied to the column and washed through in the usual manner. The ultra-violet opacity diagram (Fig. 11) obtained on gel diffusion-filtration of this substance showed pronounced 'tailing' in a manner reminiscent of the behaviour of RVF S_{low} virus (Fig. 10C). This is in striking contrast to the symmetrical curve obtained with spherical molecules such as *Jasus lalandii* haemocyanin on gel diffusion-filtration (Fig. 10A). The DNA used in these experiments showed a single sedimenting component in the analytical ultracentrifuge. The sedimentation constant found was approximately 26 Svedberg units at infinite dilution.

Effect of immune serum and nucleases

As it seemed possible that the S_{low} fraction might contain infective nucleic acid, the effects of antiserum and nucleases on its infectivity were examined. A 20 % suspension of infected mouse liver and spleen was clarified by low-speed centrifugation and the S_{low} fractions prepared from it as previously described. Neutralization tests were done on the clarified suspension and on the S_{low} fractions. Twofold dilutions of antiserum were mixed with equal volumes of test virus diluted to contain 100 to 200 LD 50 per 0·2 ml. The mixtures were left at room temperature for 6 hr. and 0·2 ml. of each was injected intraperitoneally into six mice.

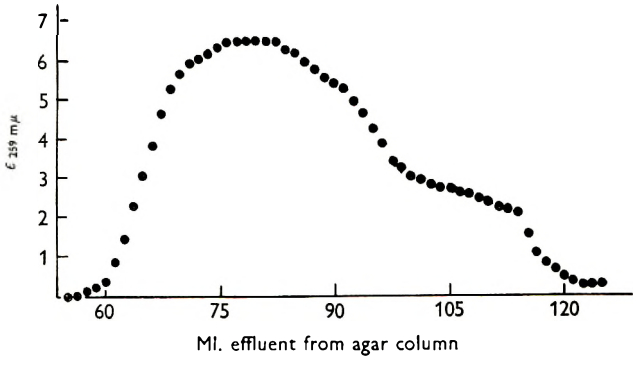

Fig. 11. Diffusion-filtration of high molecular deoxyribonucleic acid in a column of granulated 7% agar gel.

Table 3. *Effect of nucleases on the RVF* S_{low} *virus fraction*

Material tested	Negative $\log_{10}$ LD 50
RVF S_{low} control	4·15
RVF S_{low} and RNAse	3·84*
RVF S_{low} and DNAse	4·17

* The slight difference in titre between the material treated with RNAse and the untreated control is within the limits of the assay.

From the results the antiserum dilutions which protected 50 % of the mice were calculated. It was found that 100 LD 50 of the S_{low} fraction required a dilution of 1/64 and 50 LD 50 of the whole virus a dilution of 1/90. There was no evidence for the presence in either virus preparation of an unneutralizable constituent.

To test the effect of nucleases, RVF S_{low} material prepared from a liver and spleen suspension was treated with crystalline RNAse and DNAse (Seravac). Two ml. portions of the virus suspension were treated with 2 ml. of enzyme and incubated at 37° C. for 1 hr. The $MgSO_4$ (2 mg.) was added to the DNAse suspension. A control virus sample was also incubated at 37° C. The three samples were titrated in mice. The results (Table 3) show that the S_{low} material is unaffected by RNAse and DNAse.

In another experiment the S_{low} material was split into two fractions by centrifugation in a CsCl gradient. One virus fraction had a density of 1·23 g./cm.^{3} and the other consisted of the components of lower density. Each fraction was treated with both enzymes. Neither was affected by RNAse or DNAse.

DISCUSSION

Most of the infectivity of RVF virus is associated with a particle having a sedimentation constant of 452 Svedberg units (Naudé, Madsen & Polson, 1954).

It has now been shown that after repeated ultracentrifugation at a velocity sufficiently high to remove all infective virus particles from an MEF_1 poliovirus suspension (S_{20} = 156) RVF virus retains a relatively high degree of infectivity.

Cheng (1961), who determined that Semliki Forest virus has a monodisperse infective particle, also noticed traces of virus in the supernatant fluid after high-speed centrifugation, but attributed its presence to convective disturbances. If this were the case the number of infective particles in the supernatant fluid should decrease progressively during successive periods of high-speed centrifugation. This was shown to occur in experiments with monodisperse MEF_1 poliovirus when infective virus was no longer detectable in the supernatant fluid after three cycles of ultracentrifugation. However, under similar conditions, the supernatant fluids of RVF virus maintained a fairly constant infectivity. The particles responsible for this have conveniently been called the S_{low} fraction.

The experimental evidence suggests that the S_{low} virus fraction is composed of a range of particles varying in density and in sedimentation constant. The results of three experiments performed to determine the sedimentation constant of the S_{low} virus by prolonged ultracentrifugation in a *J. lalandii* haemocyanin gradient were presented. In one experiment, the infectivity of the S_{low} virus samples after centrifugation varied to such an extent that only a one-step curve was drawn (Fig. 3A), whereas in the other two experiments (Fig. 3B and C) the distribution of infectivity in the tube suggested the presence of two components. Sedimentation constants obtained from these curves (*a* and *b*, Fig. 3B and C) were about 7 and 15–19 Svedberg units.

A second method of centrifugation using the SW 39 rotor yielded a sedimentation constant of 4 Svedberg units which may be regarded as a minimum value. Although the disparity in results is within the range of experimental error of these techniques, the different sedimentation constant values obtained, together with the results of density gradient centrifugation experiments, suggest inhomogeneity in the S_{low} virus fraction.

The results of centrifugation in CsCl gradients indicated that the greater portion of the S_{low} virus has a density of 1·23 g./cm.^{3}, which is the same as that found for the readily sedimentable portion of RVF virus with a sedimentation constant of 452 Svedberg units. However, in all these experiments, the results suggested the presence of lighter components varying in density which rose to the surface in the tube during centrifugation. Owing to the method of preparing the S_{low} virus fraction (i.e. consecutive centrifugation under a layer of liquid paraffin) it is to be

expected that the virus particles of lower density would vary in amount from one S_{low} preparation to another, causing the variability in the results obtained.

It may be assumed that a smooth spherical particle of sedimentation constant 15–19 and density 1·23 g./cm.^{3} would be about 12 mμ in diameter and, when passed through a column of granulated 7 % agar, would be eluted in a similar position to *J. lalandii* haemocyanin (S_{20} = 16). In gel diffusion-filtration experiments with the denser S_{low} fraction of RVF virus, the infective zone emerged from the column with the solvent front and ahead of the largest standard protein, i.e. *Burnupena cincta* haemocyanin (S_{20} = about 90), indicating that its diffusion constant is smaller than that of either haemocyanin. The relatively low sedimentation and diffusion constants of the S_{low} virus taken together suggest a very high frictional ratio. It is therefore postulated that at least the S_{low} particles having the same density as the sedimentable RVF virus (S_{20} = 452) are filamentous. Ultrafiltration experiments showed that the S_{low} material has a filtration end-point more than double that of the S 452 form of the virus. This may be taken as further evidence for the filamentous nature of the S_{low} particles as spherical particles would filter through a membrane more readily than long filaments.

Further supporting evidence is obtained from the behaviour of high molecular DNA on the granulated 7 % agar column. This substance migrated with the solvent front and showed excessive 'tailing' in its elution curve similar to that of the S_{low} virus fraction. A possible explanation for the 'tailing' effect is that the filamentous particles were trapped in crevices in and/or between surfaces of contact of the agar granules. This is in contrast to the behaviour of spherical particles, which are delayed for shorter periods in these localities due to their higher diffusion rates. The behaviour of RVF in gel columns was shown to contrast strikingly with that of 'spherical' viruses such as MEF_1. It was also shown that the various S_{low} particles were neutralized by antibody and resistant to nucleases. They are therefore unlikely to be free infectious nucleic acid, or nucleic acid encased in a layer of non-antigenic lipoid or other material as postulated by Herriott (1961). Since most of the S_{low} virus has a density similar to that of the bulk of the virus in infective mouse serum, it may be provisionally assumed that its infectious nucleic acid is protected from nucleases in a manner similar to that of the RVF of sedimentation constant, 452 Svedberg units.

The evidence now presented suggests that certain viruses, such as Rift Valley fever, produce infective particles which are not sedimentable under conditions capable of removing most of the virus infectivity from the supernatant fluid and that these particles may be filamentous.

Gessler *et al.* (1956) reported that Rous sarcoma virus purified by their fluorohydrocarbon method was not appreciably sedimented during 20 hr. centrifugation at 144,000 *g*. in fluid of density 1·004. The supernatant fluid, which appeared to be more infective than the starting material, gave electron micrographs showing particles 10–20 mμ in diameter. These authors inferred that their treatment had broken up larger, loosely packed virus particles into infective subunits. As the density of the 'non-sedimentable' virus was not determined the cause of its resistance to centrifugation is difficult to assess. Low densities of from 1·15 to

1·19 have been assigned to Rous sarcoma virus by other authors (Kahler, Bryan, Lloyd & Maloney, 1954; Crawford, 1960) and attributed to the presence of lipoid or water in the virus particles.

The density of RVF virus (1·23 g./cm.^{3}) and that of the S_{low} virus fraction, which varies from 1·23 to < 1·1 g./cm.^{3}, is lower than the density of about 1·3 attributed to protein particles and probably indicates the presence of lipoid material in conformity with the ether sensitivity of this virus (Andrewes & Horstmann, 1949).

The S_{low} fraction, however, does not behave as a small particle so that the assumption of a filamentous shape seems the simplest explanation of its centrifuged behaviour. Those particles which rise to the surface during density gradient centrifugation may also be filamentous and presumably contain very much more lipoid than the 'normal' virus.

SUMMARY

If Rift Valley fever virus (RVF), of which the main component has a sedimentation constant (S_{20}) of about 450, is centrifuged under conditions adequate to sediment MEF_1 poliomyelitis virus (S_{20} = 156) completely, about 1 % of the original infective particles remain in the supernatant fluid. This slowly sedimenting (S_{low}) fraction was shown by centrifugation to contain infective components ranging in sedimentation constant from 4 to 19 Svedberg units. Density gradient centrifugation showed that the densities of these particles varied from 1·23 to < 1·1 g./cm.^{3}. The components were neutralized by RVF immune serum but were not affected by ribonuclease or deoxyribonuclease. In gel diffusion-filtration experiments the S_{low} virus behaved as substances having very low diffusion constants. Its filtration end-point using graded collodion membranes, is approximately two to three times higher than the 'whole' virus. It is suggested that particles comprising the S_{low} virus fraction contain lipoid material and may be filamentous.

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Viruses from the common cold. A survey in Royal Air Force recruits on arrival from civilian life

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The isolation of cytopathic agents in monkey kidney and human embryo kidney cultures from nasal washings of patients with common colds (Tyrrell & Parsons, 1960) prompted us to study common colds in R.A.F. recruits. These recruits come from many parts of the country to a central recruiting unit and any colds they have on arrival constitute a sample of infection current in different parts of the British Isles. Our aim was to assess the frequency of infection with these agents in epidemiologically unrelated colds over a period of several months from as wide an area of the country as possible.

Since this study began several papers have been published which describe the isolation of cytopathic agents from common colds. Hobson & Schild (1960) in Sheffield isolated eight strains from twenty-five patients; one strain grew in both monkey and human embryo kidney (M strain), whereas the others grew only in human kidney cultures (H strains). Tyrrell & Bynoe (1961) report the isolation of twenty-five strains from nasal washings of 110 children and adults from London, Salisbury, Sheffield, Cirencester and Epsom; Kendall, Bynoe & Tyrrell (1962) isolated eighteen agents from fifty-nine boys at an Epsom boarding school, and Higgins, Ellis & Boston (1963) isolated twenty-three strains from 428 patients in general practice in the West of England.

In the U.S.A. Hamre & Procknow (1961) isolated fifty-three strains from 199 specimens from 101 medical students with common colds. Hamparian, Ketler & Hilleman (1961) described the isolation of eighteen strains from 110 patients; and, more recently, Ketler, Hamparian & Hilleman (1962) reported the isolation of thirty-five strains from 403 adults and children.

The M strains most frequently isolated have proved similar to ECHO virus type 28 of which the JH strain of Price (1956), and strain 2060 of Pelon, Mogabgab, Phillips & Pierce (1956) were prototypes. A few other strains have been isolated which were different from ECHO 28; two such strains were reported by Taylor-Robinson & Tyrrell (1962) and one by Ketler *et al.* (1962).

H strains have been isolated more frequently than M strains but their identification has been made difficult by the fact that there are many antigenic varieties. In England, Taylor-Robinson & Tyrrell (1962) separated their strains into four different serological groups. In the U.S.A., the strains of Hamparian *et al.* (1961) belonged to six different groups; and these authors quoted the unpublished findings

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of Johnson *et al.* of at least six different serotypes among strains isolated in Washington. The thirty-four H strains described by Ketler *et al.* (1962) fell into nineteen different groups. Together these different common cold viruses, or rhinoviruses, appear to form a group with many properties similar to enteroviruses.

It has been suggested that in addition to the rhinoviruses some agents such as adenovirus, influenza and para-influenza virus, and respiratory syncytial virus, which cause acute respiratory disease in children, may be responsible for colds in adults. The role of these viruses in the common cold was also included in our study.

MATERIALS AND METHODS

Field methods

The study began at the end of May 1960 and continued for 6 months. A total of 3610 recruits were interviewed in groups of 20–30 men within 36 hr. of their arrival. All men with symptoms of acute respiratory infection were questioned and 104 with a history of onset of symptoms on the morning when seen or during the previous two days (i.e. within 56 hr.) were examined clinically. Ninety patients with typical coryzal symptoms who were afebrile at the time of examination (maximum temperature 98·4° F.) were asked if they would allow specimens to be taken for laboratory examination and all but three agreed. In addition, during visits to three R.A.F. units the opportunity was taken to obtain specimens from fifteen men who fulfilled similar clinical criteria.

Specimens

The specimens taken from each patient for laboratory study consisted of a nose or per-nasal swab and a throat swab or washing and two blood samples, one at the time and the second 2 weeks later. The swabs were broken off into screw-capped bottles containing 2 ml. of Hanks's balanced salt solution with 0·5 % lactalbumin hydrolysate (LAH) and 0·02 % sodium bicarbonate. The bottles were placed immediately in a vacuum flask containing solid carbon dioxide for transport to the laboratory, where they were transferred to an electric refrigerator and stored at −65° C. until tested. Throat washings were obtained by giving the patient 10 ml. of 10 % nutrient broth-saline and asking him to gargle for 1 min. The washings were collected into sterile 20 ml. screw-capped bottles and transported on solid carbon dioxide to the laboratory where they also were stored at −65° C.

A sample of blood was obtained from all the patients in the acute stage of illness, but convalescent specimens were taken from only eighty-eight patients, the remaining fourteen having left the Service. At the time of the second bleeding details were recorded of the duration of symptoms, the presence of any complications and history of other respiratory illnesses since the first interview. Serum specimens were stored in the laboratory at −30° C. until they were tested.

Laboratory methods

Isolation of virus

Monkey kidney and human embryo kidneys were trypsinized and grown in Hanks's saline with 0·5 % LAH and 5 % ox serum. Before inoculation the medium was altered to contain 2 % calf serum and 0·03 % bicarbonate.

HeLa cells were grown in Gey's solution with 0·5 % LAH and 10 % human serum and tube cultures were maintained with 0·25 % LAH + 5 % rabbit serum.

Tubes of each tissue were inoculated with 0·1 ml. amounts of the materials to be tested, the HeLa cell cultures were incubated without rolling at 37° C., whereas monkey and human embryo kidney cells were kept rolling at 33° C.

Cultures were examined on alternate days for evidence of cytopathic change. Fluids were replaced with fresh medium when necessary and incubation was continued for a period of 3 weeks or longer if the cell sheets were still healthy. Once cytopathic changes were noted, subcultures were made and pools of infected fluids prepared and titrated in readiness for neutralization tests.

Preparation of rabbit antisera

Rabbit antisera were prepared with the prototype H strain FEB (Sal/1/58/H) and the prototype M strain HGP (Sal/1/57/M) by repeated twice-weekly intravenous inoculations. The first H strains isolated were tested with the H prototype antiserum and, as only one was neutralized by it, rabbit antisera were prepared with some of them. These antisera were prepared by twice-monthly intramuscular injections of virus with a Bayol–Arlacel adjuvant for a period of 3 months. Rabbits were bled after a further 3 weeks. Sera were inactivated by heating at 56° C. for $\frac{1}{2}$ hr. before use.

Neutralization tests

Rhinovirus M strains were identified by serum neutralization tests. Equal volumes of serum dilutions and virus suspensions containing 200 TCD 50/ml. were kept at room temperature for 1 hr. and then transferred to monkey kidney cultures and incubated at 33° C. Results were read 2 days after the virus controls showed a definite cytopathic effect. Neutralization end-points were recorded as the highest serum dilution preventing a cytopathic effect.

For the estimation of antibodies to ECHO 28 and HGP viruses, acute and convalescent sera were tested first in a dilution of 1/5 followed by titration of any pairs showing antibody.

Rhinovirus H strains were identified by a method based on the reduction of microplaques as described by Taylor-Robinson & Tyrrell (1962) with a dose of virus estimated to produce between 10 and 50 microplaques per tube in 2–3 days.

For the estimation of antibodies to FEB virus, acute and convalescent sera were tested in a similar manner with a dilution of 1/10. Paired sera from patients from whom strains of virus were isolated were tested for antibody response to the infecting strain.

For the detection of antibodies to Coxsackie A 21 (Coe virus) paired sera were tested by neutralization in HeLa cells as described by Pereira & Pereira (1959).

Complement-fixation tests

These were done in plastic haemagglutination trays with 0·1 ml. unit volumes and overnight fixation at 4° C. Antigens were standardized by chess-board titration with human convalescent sera and used at optimal dilution. The antigens used were: influenza A, B and C, para-influenza 1, 2 and 3, adenovirus and respiratory syncytial virus. An antigen prepared with Eaton agent was kindly supplied by Dr B. E. Andrews.

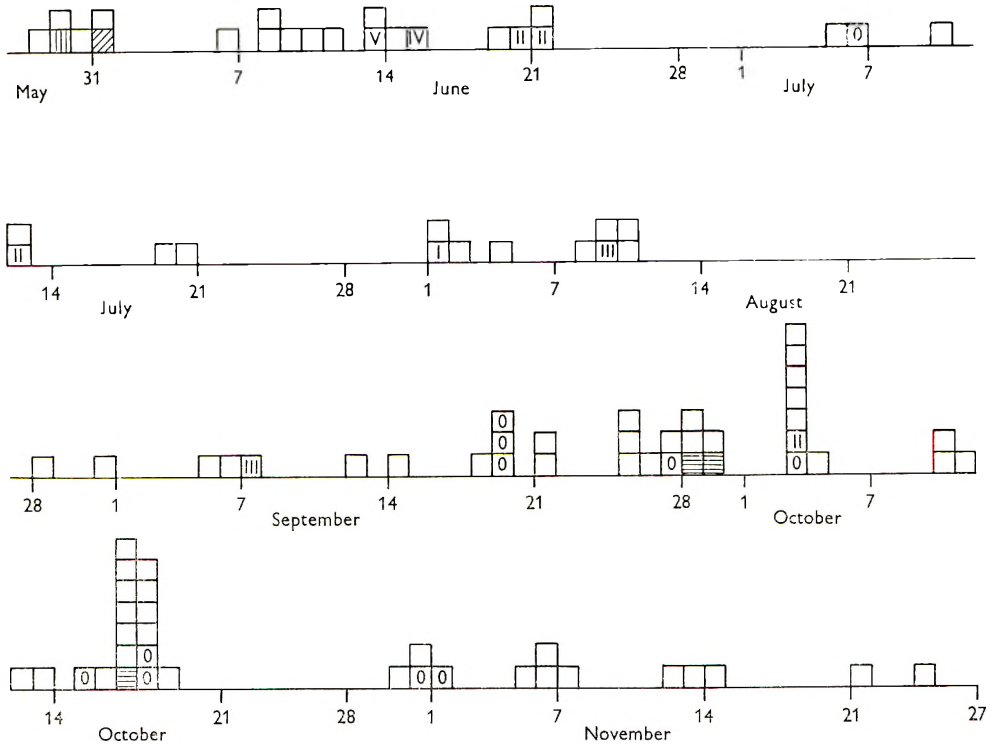

Fig. 1. Common colds in an R.A.F. recruit centre, June–November, 1960. Each square indicates a cold from which specimens were tested. Virus infections are shown as follows: ECHO 28, RS, Influenza C. Rhinovirus H by numbers I–V or 0 (unclassified) corresponding to serological groups in Table 1.

RESULTS

Virus isolation

Altogether twenty-three strains of virus were isolated from the 102 patients examined. All these agents produced a cytopathic effect in human embryo kidney cultures and three of them in monkey kidney cultures also. None of the specimens, including those that yielded these twenty-three strains, caused detectable effects in HeLa or human amnion cultures.

Virus strains were isolated from 19/99 (19 %) nasal or per-nasal swabs and from 10/102 (10 %) throat swabs or washings. Strains were isolated from 3/12 (25 %) specimens taken within 24 hr. of the onset of illness, from 11/43 (26 %) specimens

taken between 24 and 48 hr., and from 9/47 (19 %) specimens taken between 48 and 56 hr.

The seasonal distribution of the colds from which agents were obtained is shown in Fig. 1. The H strains were isolated from material from four patients with typical common colds in June, two patients in July, two in August, five in September, six in October and one in November. Six strains were from patients coming from the south of England and Wales, seven from the Midlands, one from the north of England, and five from Scotland and Ireland. One man had just arrived from Fiji. No particular geographical pattern was evident.

Table 1. *Cross-neutralization tests with rabbit antisera to 'H' strains of rhinovirus*

	Rabbit antiserum						
	I		II		III	IV	V
Rhinovirus strain	Sal/1/58/H FEB	T	W	Mor	Md	Mu	L
Sal/1/58/H FEB	+	+	–	–	–	–	–
T	+	+	–	–	–	–	–
Sheffield/1/60/H 16/60	–	–	+	+	–	–	–
W	–	–	+	+	–	–	–
Mor	–	–	+	+	–	–	–
P	–	–	+	+	–	–	–
B	–	–	+	+	–	–	–
Md	–	–	–	–	+	–	–
F	–	–	–	–	+	–	–
Mu	–	–	–	–	–	+	–
L	–	–	–	–	–	–	+
Sal/1/59/H Th	–	–	–	–	–	–	–
Sal/1/51/H No	–	–	–	–	–	–	–
N, Mur, O'L, S, Mg, C, D, Bo, R, Ru, Br	–	–	–	–	–	–	–

+ = K value > 0·2. – = K value < 0·2.

Identity of virus strains

The three M strains were identified as ECHO 28 virus on the basis of neutralization by a monkey antiserum to this virus. Virus was isolated from all the specimens (nasal or per-nasal swabs, throat swabs and gargles) obtained from the three patients in question. All three men came from the south of England but they were not apparently associated in any way; their colds occurred between the end of September and the middle of October.

The twenty H strains isolated were tested with a rabbit antiserum prepared with the FEB virus (Sal/1/58/H), the only named or known H strain available to us. As only one strain was found to be neutralized, further antisera were prepared in rabbits with some of the other strains. The results of cross-neutralization tests with all available antisera are shown in Table 1.

From the absence of antigenic crossing in these tests it can be seen that there were at least six different antigenic types prevalent during the survey. The group numbered II contained the largest number of similar strains. Of the four strains

in this group two were from colds during the same week in June, one from a cold in July 3 weeks later and one from a cold in October. The four patients came from widely separated places.

Serological tests

All three of the patients from whom ECHO 28 virus was isolated responded to infection with a fourfold or greater increase in neutralizing antibody to this virus. None had detectable antibody in a 1/5 dilution of the acute-stage sample of serum.

Paired sera were obtained from fourteen of the men from whom H strains were isolated. Of these only six showed an increase in neutralizing antibody to the virus isolated. The possibility of accidental infection of cultures in the laboratory was investigated by returning to the original throat or nose washings and attempting re-isolation. In every case this was successful.

The proportions of patients with neutralizing antibodies in their acute-stage specimens to the prototype M strain HGP (Sal/1/57/M), to the H strain FEB (Sal/1/58/H), and to ECHO 28 were 89, 45 and 35 %, respectively. There was no appreciable difference in the proportion with antibody against these viruses in men under or over 20 years of age. However, the age range in population studied was narrow and all but two were aged between 17 and 28 years.

The association of rhinoviruses with twenty-three common colds still left a large number from which no agent was isolated.

All the results of serological tests with other antigens were negative except for one pair showing a fourfold increase to influenza C and one pair to respiratory syncytial virus. Both these patients had complement-fixing antibody in the acute-stage serum, suggesting either that infection occurred despite the presence of antibody or that it was coincidental and not the cause of the cold.

Table 2. *Clinical picture on the first day of illness*

Symptoms	Virus isolated (23 patients) (%)	No virus isolated (79 patients) (%)
Coryza (clear nasal discharge)	74	89
Blocked nose	61	41
Dry sensation in throat or slight sore throat	43	39
Slight hoarseness	22	8
Dry cough	4	6
Sweating	9	4
Shivering	9	1

Clinical findings

The symptoms present in the 102 patients who took part in this investigation were fairly typical of the mild afebrile upper respiratory illness usually associated with the common cold. The distribution of symptoms in patients from whom viruses were and were not isolated is shown in Table 2; there appeared to be no important differences. The three illnesses associated with ECHO 28 infection were also similar to the rest.

DISCUSSION

The role of respiratory viruses other than rhinoviruses in the causation of common colds was found to be almost negligible in this group of R.A.F. recruits studied during the summer and autumn months. These findings presumably reflect the fact that during these seasons most other respiratory viruses are seldom epidemic and contrast with results obtained in a preliminary survey made during the previous winter. During that period febrile respiratory illness was prevalent and associated with infection with adenovirus type 3, Coxsackie A 21 (Coe virus) and, to a lesser extent, para-influenza 3 virus. One or other of these viruses was isolated from 7/23 afebrile common colds which were concurrently investigated, but no rhinoviruses were isolated. The results of this survey do not allow one to compare the prevalence of rhinoviruses in autumn and winter, but it has been our experience that rhinoviruses are infrequently found associated with common colds in winter and it may be that this group of viruses tends to follow the seasonal pattern of other enteroviruses.

The rhinoviruses isolated in the present study were a heterogeneous collection. The ECHO 28 strains grew relatively quickly to a high titre in monkey kidney cultures and the patients developed antibody. The twenty H strains grew rather slowly and needed several passages before workable titres of virus were obtained. Titres above 10^4 were exceptional. Less than half the patients developed antibody against the strains isolated but the convalescent sample of serum was taken 2 weeks after the onset of illness and it is possible that specimens taken later would have contained antibody.

The prevalence of antibody against the three prototype rhinoviruses HGP, FEB, and ECHO 28 as judged by results of tests on acute-stage sera suggest that a high proportion of people have been infected with these agents and possibly reinfected. It is remarkable that 45 % had antibody to the H strain FEB having regard to the fact that antibody was not always detected even after the isolation of a strain of virus. The M strains appear to be better antigens so that the figure of 89 % for HGP reflects a high prevalence of this strain at some time in the past. The figure of 35 % for ECHO 28 is of the same order as that found by Price, Emerson, Ibler, Lachaine & Terrell (1959) who reported the presence of neutralizing antibody in 5 % of children under 5 years of age rising to 35 % in the 6–12 age group and to 63 % in the 25–35 age group.

The number of distinct serological types of rhinovirus found associated with this small group of colds indicates the complexity of the disease.

The prevalence of certain types may vary in time and it is significant that the group containing the largest number of similar strains was one in which the prototype strain was isolated in the same year.

At least three different antigenic types of virus were defined in addition to those already identified in this country. Neutralization tests with rabbit antisera to these three, designated Md, Mu and L, against five different serotypes from the National Institutes of Health, U.S.A., designated 33342, 353, 1059, 11757 and 1734 (Johnson & Rosen, 1963), have shown them to be antigenically distinct.

SUMMARY

Twenty-three strains of rhinovirus were isolated from 102 patients who had common colds on arrival at a Royal Air Force recruit centre during a 6-month period from June to November, 1960. Three of these strains were M type rhinoviruses similar to ECHO 28 virus. Twenty strains were H type rhinoviruses which fell into six or more different antigenic types. Two of these types were similar to the prototypes Sal/1/58/H and Sheffield/1/60/H. Three types were antigenically distinct from those previously reported in this country and several strains are still unclassified. Other human respiratory viruses were not isolated from common colds occurring at this time among the population studied.

We are indebted to the Senior Medical Officer of R.A.F. Cardington for providing facilities for the investigation, to Squadron Leader M. Shearer for his help in collecting convalescent blood specimens, and to the Director-General of the Royal Air Force Medical Service for permission to publish this paper.

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The typing of enteroviruses in tissue culture by neutralization with composite antiserum pools

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Because of the many known types of enterovirus in existence (Report, 1962) identification of newly isolated strains by specific neutralization is becoming increasingly difficult. Neutralization tests with individual type specific antisera are so uneconomical in tissue culture and time that the use of pooled antisera initially is advantageous.

The preparation of antiserum pools, in which a given antiserum is included in more than one pool, so that strains can be identified from the pattern of positive results obtained in a single set of neutralization tests, has already been reported (Lim & Benyesh-Melnick, 1960; Schmidt, Guenther & Lennette, 1961). Although results are obtainable fairly quickly such methods are expensive in their use of antisera.

In the scheme which is now described virus identification is done in two stages. The identity of a virus is established less rapidly than in the other methods referred to, but the scheme has the advantages of economy in tissue culture and sera, of easy extension to cover more viruses and of allowing each virus to be neutralized by a specific antiserum on two separate occasions. It appears to work satisfactorily on most occasions but any uncertain results necessitate repetition and, if necessary, full neutralization tests with individual antisera. The methods of preparation of the composite antiserum pools and the procedures for virus typing are first described. They are followed by some results of virus identification by this typing scheme.

PREPARATION OF THE ANTISERUM POOLS

The scheme described provides for the identification of twenty-seven viruses: polioviruses 1–3; Coxsackie viruses A 9 and B 1–6; ECHO viruses 1–3, 5–7, 9, 11–16, 19, 22–24. The antisera were prepared in rhesus monkeys or rabbits by immunization with prototype viruses. The titre of an antiserum, based on a volume of 0·1 ml., was the highest dilution which would neutralize about 100 tissue-culture doses (TCD 50) of the homologous prototype virus, when this was suspended in 0·1 ml. of maintenance medium. The amount of serum in such a dilution then constituted one unit of antibody. In the titrations 0·3 ml. of serial fourfold dilutions of serum were mixed with 0·3 ml. of virus suspension containing about 300 TCD 50. After remaining 1 hr. at room temperature 0·2 ml. volumes of the mixtures were inoculated into duplicate tubes of rhesus monkey kidney cultures.

Control titrations to check the correctness of the virus dose were made at the same time. Cultures were incubated at 36–37° C. and examined microscopically at intervals. Titres, based on the neutralization of the cytopathic effect (C.P.E.), were calculated from the readings on the sixth day as 50 % end-points by the method of Kärber (1931). All titres quoted refer to initial serum dilutions before the addition of virus. All the sera were tested for neutralizing antibody to the other viruses in their group. Only those with little or no heterologous antibody were used.

Table 1. *Enterovirus pool* 1

Antiserum	Serum titre	Dilution containing about 50 units/0·1 ml.	Dilution for use in the pool
Poliovirus 1	1,280	1/25	1/8
Poliovirus 2	10,000	1/200	1/67
Poliovirus 3	2,560	1/50	1/17

Table 2. *The four major composite antiserum pools*

Enterovirus pool (EP) number	Antisera contained in the pools
EP 1	Polioviruses 1–3
EP 2	Coxsackie B 1–6
EP 3	ECHO 1–3, 5–7, 9, 11 and Coxsackie A9
EP 4	ECHO 12–16, 19, 22–24

Table 3. *Association of* EP 3 *with pools* A–F

Pool identity	Antisera in pool
A	ECHO 1, 5, 9
B	ECHO 2, 6, 11
C	ECHO 3, 7, Cox A9
D	ECHO 1, 2, 3
E	ECHO 5, 6, 7
F	ECHO 9, 11, Cox A9

Table 4. *Association of* EP 4 *with pools* G–M

Pool identity	Antisera in pool
G	ECHO 12, 15, 22
H	ECHO 13, 16, 23
J	ECHO 14, 19, 24
K	ECHO 12, 13, 14
L	ECHO 15, 16, 19
M	ECHO 22, 23, 24

The composition of the pools was designed so that about 50 units of each antiserum were present in 0·1 ml. of a pool. In making the pools equal volumes of all the sera were mixed, the requisite initial serum dilutions being obtained by multiplying that dilution which contains 50 units by the number of sera in the pool. As an example Table 1 shows how enterovirus pool 1 was prepared.

Serum dilutions were made in Earle's balanced salt solution containing sodium bicarbonate 0·18 %, penicillin 100 units/ml. and streptomycin 100 μg./ml. The serum pools, distributed in small amounts, were stored at −30° C. until required. For use one container was thawed and kept at 4° C. until finished. No deterioration in titres was observed over periods of 3 months.

For the scheme four major pools of antiserum, labelled enterovirus pool (EP) 1–4 and twelve secondary or intersecting pools in alphabetical order A–M (excluding I)

were prepared. The composition of the major pools is shown in Table 2, pool 1 being concerned with the polioviruses, pool 2 with the Coxsackie B viruses, and pools 3 and 4 with the ECHO and Coxsackie A 9 viruses. The largest number of distinct serotypes occurs among the ECHO viruses, therefore the secondary pools—six linked with EP 3 and six with EP 4—are used in the identification of these agents. This association is shown in Tables 3 and 4.

VIRUS IDENTIFICATION

Stationary culture tubes ($4 \times \frac{1}{2}$ in.) of primary rhesus monkey kidney, primary human amnion and HeLa cells incubated at 36–37° C. have been used for virus identification. Other types of cell and sizes of tubes may be found just as satisfactory. The susceptibility of the different cells may be a guide to the identity of an unknown virus, which should be typed in the culture system found to be most susceptible. The virus suspension for use in the neutralization tests is prepared by the inoculation of cultures of the specified cells. After inoculation the cultures are examined daily for cytopathic changes. Complete destruction of the cell sheet within 3 days is preferable, and if this is not obtained initially a further passage should be made. When destruction is complete, the cultures are frozen at −30° C., rapidly thawed at 37° C., and harvested. This harvest forms the stock virus suspension for all the neutralization tests and is stored at −30° C. until required. Under these circumstances the approximate titres of the stock suspensions may be estimated as depicted in Table 5. Experience has shown that, within limits, the dilutions suggested are usable without the need for detailed titrations.

Table 5. *Estimation of virus titres*

Time for complete CPE to occur	Approximate infective virus TCD 50/0·1 ml.	Dilution of stock virus suspension for tests
1 day	10^6	10^{-4}
2 days	10^5	10^{-3}
3 days	10^4	10^{-2}

When a suitable suspension of the unidentified virus is available and a decision is made on the cell cultures to be used, the virus is tested against the major pools of antiserum (EP 1–4). Inhibition of the cytopathic agent by a pool places the unknown virus in one of the four groups. In the tests 0·1 ml. of virus suspension, diluted to contain an estimated 100 TCD 50 of virus (Table 5), is mixed with 0·1 ml. of each of the antiserum pools. After 1 hr. at room temperature, 0·1 ml. of each serum-virus mixture is inoculated into one tissue-culture tube containing 1 ml. of maintenance medium. As neutralization of virus growth is expected in only one culture the other three act as virus controls. The final readings are made on the sixth day.

Once allocated to a group a virus may be identified by the same technique of neutralization with the appropriate antisera in the following manner. For the first group the individual polioviruses 1–3 antisera may be used and for the second

group the individual Coxsackie B viruses 1–6 antisera. For the third group the intersecting ECHO and Coxsackie A 9 antiserum pools A–F, and for the fourth group pools G–M are used. Examples of how the system of neutralization works with pools A–M are shown in Fig. 1 (*a*) and (*b*).

(*a*) Pools A–F

	A	B	C
D	Echo 1	Echo 2	Echo 3
E	Echo 5	Echo 6	Echo 7
F	Echo 9	Echo 11	Cox. A9

(*b*) Pools G–M

	G	H	J
K	Echo 12	Echo 13	Echo 14
L	Echo 15	Echo 16	Echo 19
M	Echo 22	Echo 23	Echo 24

Fig. 1. Neutralization patterns obtained with intersecting antiserum pools. ▥, Neutralization by pools B and E indicates that the agent is an ECHO 6 virus. ▤, Neutralization by pools J and K indicates that the agent is an ECHO 14 virus.

RESULTS

To assess the value of the scheme a series of 115 viruses was tested. All the reported experiments were done in stationary cultures of primary rhesus monkey kidney cells maintained with medium 199 containing 0·22 % sodium bicarbonate, penicillin and streptomycin. The technique of neutralization has been described. The series consisted of ninety-six recently isolated viruses previously identified by standard methods, seventeen prototype strains and two unidentified strains as shown in Table 6A and B. Of ninety-seven viruses (Table 6A) which could have been identified eighty-seven (90 %) were successfully typed at the first attempt. Later, in repeat tests, some of the remaining viruses (Table 6A) were successfully identified by the use of a smaller dose of virus. Of the eighteen viruses (Table 6B) which did not have their corresponding antisera in the pools none were neutralized. Thus no false positive identification results were obtained.

DISCUSSION

The use of composite pools of antiserum and only a single tissue-culture tube for each serum-virus mixture is essentially a minimal screening method in the attempted identification of a large proportion of newly isolated enteroviruses. This scheme differs from the methods already reported. In particular complete virus identification requires two stages instead of one. Each stage consists of a set of neutralization tests; the first places the virus in one of the four main groups (EP 1, 2, 3 or 4) and the second establishes the precise type. Although a two-stage method has the disadvantage that it takes up to 12 days to identify a virus, it is favoured by an economy in the use of tissue cultures and by its minimizing the wastage of antiserum. It also includes the important safeguard of allowing each virus to be neutralized by a specific antiserum on two separate occasions before final identification.

Table 6. *Results of neutralization tests with composite antiserum pools*

	Virus*	No. tested	No. identified	No. not identified
A. Viruses typable by the composite antiserum pools	Poliovirus type 1	3†	2	1
	Poliovirus type 2	1†	1	—
	Poliovirus type 3	3†	2	1
	Coxsackie B1	1†	1	—
	Coxsackie B2	1	1	—
	Coxsackie B3	1†	1	—
	Coxsackie B4	6	5	1
	Coxsackie B5	2	2	—
	Coxsackie B6	1†	1	—
	Coxsackie A9	8	8	—
	ECHO 1	11	11	—
	ECHO 2	3	3	—
	ECHO 3	1†	1	—
	ECHO 5	2	2	—
	ECHO 6	13	11	2
	ECHO 7	2	2	—
	ECHO 9	2	2	—
	ECHO 11	5	4	1
	ECHO 12	3	3	—
	ECHO 13	3†	3	—
	ECHO 14	10	10	—
	ECHO 15	2†	1	1
	ECHO 16	2†	1	1
	ECHO 19	8	6	2
	ECHO 22	1	1	—
	ECHO 23	1†	1	—
	ECHO 24	1†	1	—
	Totals	97	87	10
B. Viruses not typable by the composite antiserum pools	ECHO 4	2	—	2
	ECHO 17	2†	—	2
	ECHO 18	1†	—	1
	ECHO 20	2†	—	2
	ECHO 21	2	—	2
	ECHO 25	3	—	3
	ECHO 26	1†	—	1
	ECHO 27	1†	—	1
	Adenovirus type 1	2	—	2
	Unidentified‡	2	—	2
	Totals	18	—	18

* Previously identified by neutralization with individual antisera.
† Including one prototype strain.
‡ Not neutralized by antisera to ECHO 1–28, Coxsackie A7, A9, B1–6, polioviruses 1–3.

In its present form the scheme can be used for the identification of twenty-seven enteroviruses, but it can be extended to cover more viruses with only a very small increase in the tissue-culture requirement. For example, the preparation of a fifth major pool and its corresponding intersecting pools could provide coverage for a further nine enteroviruses and yet only increase the tissue culture demand by a single tube.

The scheme is flexible in that, if the identity of the virus is suspected from its growth characteristics in different cell cultures, some or all of the major pools may be omitted and the virus may be typed with the intersecting pools or the individual antisera. An important requirement is the use of high titre antisera in the pools. This is to ensure that the necessary initial concentration of a serum which is used to provide 50 units of antibody in the final mixture does not contain any heterologous antibody. Nor should it be likely to inhibit viruses non-specifically thereby giving false positive results. In the results reported these requirements have been met.

In the tests, although an estimated 100 TCD 50 of virus in 0·1 ml. is mixed with an equal volume of serum only half of this serum–virus mixture is transferred to the culture tube. Thus if no neutralization occurs only about 50 TCD 50 of virus is introduced into the culture; however, this should be sufficient to demonstrate cytopathic change. Although theoretically the whole 0·2 ml. of mixture should be inoculated into the culture a measured 0·1 ml. amount gives satisfactory results. The amount of virus suggested for use in virus–serum mixtures is an estimate, which may well err on occasions. Failure of specific inhibition of a virus may be due to too great a virus concentration or to a mixture of viruses—a problem this scheme does not solve. It may also be because the unidentified viruses are prime strains (Melnick, 1958) which have a broader antigenic structure and are therefore not neutralized to the same extent by antiserum to the prototype viruses.

As this method is essentially a screening procedure to identify many viruses and to allow those not readily recognized to be examined more thoroughly, it is important that no false positive results should be obtained. The results show that during the testing of 115 viruses no agent was wrongly identified.

SUMMARY

A scheme is described for the identification of enteroviruses in tissue culture by neutralization with composite antiserum pools. The method for making these pools is given. Antisera to twenty-seven enteroviruses were included in the pools which were used to examine 115 viruses consisting of ninety-eight recently isolated viruses and seventeen prototype strains. The results indicate that this scheme provides a useful screening method for identifying enteroviruses. It has proved to be practicable, time saving and very economical in tissue culture.

We are grateful to Dr C. M. P. Bradstreet who made available some of the rabbit antisera.

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J. Hyg., Camb. (1963), **61**, 485
With 1 *plate*
Printed in Great Britain

Growth, toxigenicity and virulence of *Pseudomonas aeruginosa*

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More than 80 years has elapsed since the first recognition of *Pseudomonas aeruginosa* as a human pathogen (Gessard, 1882), and its importance has increased considerably in the last decade because of its antibiotic resistance. The species as a whole has been known to be an opportunist which invades the host tissue and establishes infections most readily when the host is already weakened by other causes, such as burn, malignancy or diabetes mellitus. The factor which determines the virulence of the organisms, however, is very little understood. In a previous study (Liu, Abe & Bates, 1961) various products of this species were separated into five fractions and their roles in the pathogenesis were elucidated. This work was done with two virulent strains of this species and the question as to what determines the virulence of *Ps. aeruginosa* was left unanswered.

The conventional methods for the study of the virulence of bacteria usually express the mortality of animals injected with the organisms. The results are presented numerically, either as the number of animals that died or as the numbers of bacteria that were required to kill 50% of the animals, i.e. the LD 50. These methods, however, are grossly inadequate in the study of the virulence of *Ps. aeruginosa* for several reasons. First, the organisms usually produce localized infections, such as wounds of skin, otitis media and urinary tract infections, and sepsis that results in the death of the host is rare. Death from sepsis of experimental animals, therefore, does not really express the true mode of action of *Ps. aeruginosa*. Secondly, the virulence of this species is usually very low compared with that of other pathogens, such as *Salmonella* or *Pasteurella* and, therefore, the relatively large number of organisms required to cause death may result in the toxic death of the host, without showing the true virulence of the the organisms, which includes invasiveness as well as toxigenicity. Thirdly, the resistance of mature man and animals to the infections of *Ps. aeruginosa* appears to be quite high owing to the presence of antibodies probably acquired as the results of latent infections; therefore, the data of mortality of animals infected with this organism are influenced by the resistance of the hosts as well as the virulence of the organisms. It was decided, therefore, to study the virulence of *Ps. aeruginosa* by the development of skin lesions in the same individual animal.

In order to establish infection in a host it is obvious that the organisms must be able to grow in the tissue of the host. A study in this direction has already been made by Colebrook, Lowbury & Hurst (1960) who found that some strains of *Ps.*

* Recipient of a medical student summer research fellowship.

aeruginosa were able to grow in fresh human serum but others were not able to do so. This study, however, left unanswered the question of what determines the virulence, because it was not possible to show that the ability of this organism to grow in human serum was related to virulence of man. As pointed out previously from this laboratory (Liu *et al.* 1961), the cells of *Ps. aeruginosa* are relatively non-toxic, and, therefore, even if the organism can grow freely in the host tissue the growth of this type of non-toxic cells alone is unlikely to explain the pathological picture produced by this infection. On the other hand, we have found that many strains of *Ps. aeruginosa* that were able to produce considerable amounts of extracellular toxic substances, such as haemolysin, lecithinase and protease, were quite non-virulent when injected intracutaneously into rabbits, i.e. they failed to produce the lesions which were produced when the toxins formed *in vitro* were injected. In order to evaluate the relative importance of toxigenicity and ability to grow in the host tissue in the manifestation of virulence, a large number of strains of *Ps. aeruginosa* were tested for their ability to grow in fresh serum of rabbits. The virulence of these organisms was also tested in the same rabbits from which the sera were obtained. The results were then compared with the ability of the organisms to produce various extracellular toxic substances *in vitro*. The present communication describes one of these experiments which appears to throw some light on the factors that determine the virulence of *Ps. aeruginosa*.

MATERIALS AND METHODS

Four groups of *Ps. aeruginosa* were selected for this study. The first group, represented by strains P-A-7 and P-A-214, was the type which grew readily in fresh rabbit serum and produced large amounts of haemolysin, lecithinase and protease, which will be referred to collectively as the extracellular toxins (ET). The second group, represented by the strain P-A-4, was the type which produced considerable amounts of ET but failed to grow in the fresh rabbit serum. The third group, represented by the strain 1419, was the type which grew readily in fresh serum but failed to produce appreciable amounts of ET. The fourth group, represented by the strains 117 and 1184, was that which failed to grow well in fresh serum and also failed to produce appreciable amounts of ET. All the 6 strains described here were isolated from human sources.

The blood of the rabbits was obtained from the marginal vein of the ear and the serum was Seitz-filtered. The growth of the organisms in the sera as well as in the trypticase soy broth (Baltimore Biological Laboratory) was estimated by inoculating a sufficient number of cells to make the initial concentration to about 10^5 per ml., taking samples of the cultures (37° C.) at intervals, making tenfold dilutions in saline, and then plating 0·1 ml. of each dilution in duplicate on trypticase soy agar. The average of the colony counts of the two plates was taken to express the rate of growth. The virulence of the organism was observed by the development of a lesion in the back of the rabbit after intracutaneous injection of 0·1 ml. of an 18 hr. broth culture. The production of the ET *in vitro* and their titrations were carried out as described previously (Liu *et al.* 1961).

RESULTS

In Text-fig. 1 are shown the growth curves in trypticase soy broth of the 6 strains of *Ps. aeruginosa*. The strains P-A-7, P-A-214, P-A-4 and 1419 did not show significant differences in their rates of growth in this medium. The strains 117 and 1184 were definitely slower than the other 4 strains in their rates of growth. The titres of haemolysin and extracellular enzymes produced by these organisms are listed

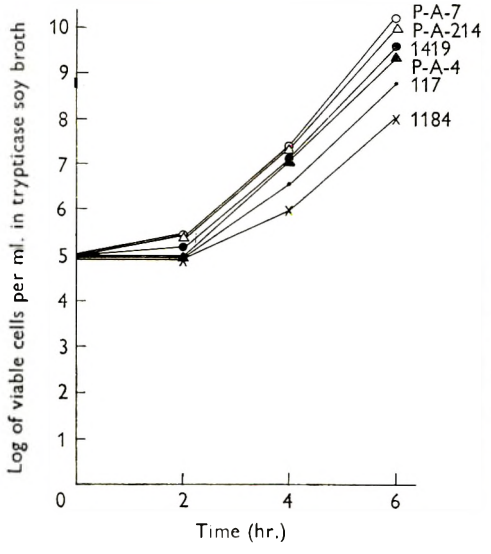

Fig. 1

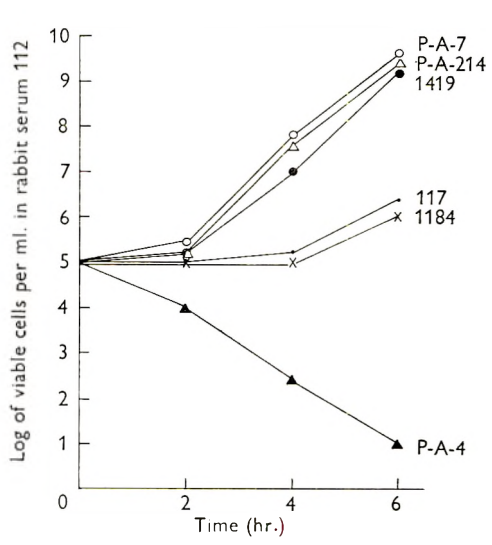

Fig. 2

Text-fig. 1. Growth curves of 6 selected strains of *Ps. aeruginosa* in trypticase soy broth.

Text-fig. 2. Growth curves of the same 6 strains of *Ps. aeruginosa* in the fresh serum of rabbit no. 112. Strains P-A-7, P-A-214 and 1419 grew well in this serum while 117 and 1184 hardly grew at all. Strain P-A-4 appeared to have been killed by this serum.

Table 1. *Toxigenicities* in vitro *of six selected strains of* Pseudomonas aeruginosa

Strain	Haemolysin	Lecithinase	Protease
117	8	0	8
1184	< 4	0	4
1419	< 4	0	8
P-A-4	32	16	64
P-A-7	64	32	128
P-A-214	32	32	128

Reciprocals of the highest dilutions of the crude preparations showing a complete reaction are listed as the titres.

in Table 1. Strains P-A-4, P-A-7 and P-A-214 produced considerable amounts of these extracellular toxic substances, but strains 117, 1184 and 1419 were very poor producers. The growth curves of these 6 strains in the serum of rabbit no. 112 are shown in Text-fig. 2. As will be seen in this figure, the strains P-A-7, P-A-214

and 1419 grew readily while the other 3 strains did not. One-tenth ml. of 18 hr. broth cultures of these 6 strains of *Ps. aeruginosa* were then inoculated intradermally into rabbit no. 112 from which the serum was obtained. In Pl. 1 are shown the results 48 hr. after the inoculation. Strains P-A-7 and P-A-214 produced large areas of induration and redness of skin; considerable necrosis was noted also in the centre of the lesions. These findings were expected because both of these strains grew well in the serum of this rabbit and they produced large amounts of ET. Strains 117 and 1184 failed to show any lesion. These findings were also expected because neither of these strains grew well in the serum of this rabbit and they did not produce ET in significant amounts. The most interesting part of this experiment, however, was the behaviour of the strains P-A-4 and 1419. The strain 1419 grew well in this serum but failed to produce significant amounts of ET. The strain P-A-4, on the contrary, produced considerable amounts of ET but failed to grow in this serum. It was hoped that the behaviour of these two strains, representing two groups of *Ps. aeruginosa*, would provide an answer to the question whether the toxigenicity or the ability to grow in the sera of animals would constitute the determinant factor of the virulence of *Ps. aeruginosa*. As shown in Pl. 1 neither strain P-A-4 nor 1419 was able to produce a significant lesion in the skin of rabbit no. 112. It appears, therefore, that neither the ability to grow in the sera of animals, nor the ability to produce various extracellular toxic substances alone is sufficient to make a strain of *Ps. aeruginosa* virulent to animals. In other words, a strain of *Ps. aeruginosa* must be able both to grow in the serum of an animal and to produce various types of extracellular toxins in order to be virulent to that animal. Although all the rabbits used in this study were supposed to be normal animals that had never been used in any experiment, considerable differences in pathological pictures were observed from one rabbit to another using the same group of *Ps. aeruginosa*. The differences were usually found among the group of *Ps. aeruginosa* which were able to produce large amounts of ET, in which the virulence of the organisms appeared to be a function of their ability to grow in the sera of animals. Those strains which failed to produce appreciable amounts of ET were usually nonvirulent regardless of their ability to grow in the sera of animals, and, therefore, the results were consistent from one experiment to another. The ability of a serum sample to inhibit the growth of a strain of *Ps. aeruginosa* appeared to depend largely on the presence of a type-specific antibody, because the inhibitory effect of the serum could be absorbed out by cells of the susceptible strains of *Ps. aeruginosa*, but not by those of the strains which grew readily in the same serum. These findings were not surprising because of the ubiquitous nature of *Ps. aeruginosa*. Many of the so-called normal animals one purchases from animal dealers were probably exposed to some strains of *Ps. aeruginosa* at some time in their life span. The presence of *Ps. aeruginosa* antibodies in human sera has been described by many workers (Gaines & Landy, 1955; Whitby, Michael, Woods & Landy, 1961). These specific antibodies which exist in the sera of animals and man have most likely been produced by exposure of the individuals to latent infections. In order to circumvent this possibility we have tested many samples of human placental serum for their ability to inhibit the growth of *Ps. aeruginosa*. Text-figs.

3 and 4 show the growth curves of the 6 strains of *Ps. aeruginosa* with two samples of placental sera. As will be seen in these figures, sample A permitted the growth of strains P-A-7, 1419, 1184 and 117 but inhibited the growth of P-A-4 and P-A-214. Sample B, on the other hand, permitted the growth of P-A-214 but inhibited 117.

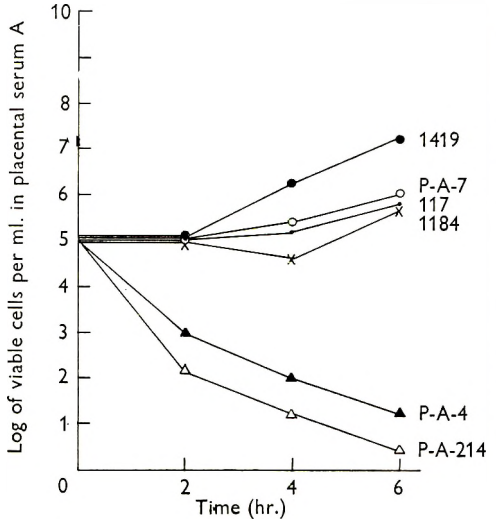

Fig. 3

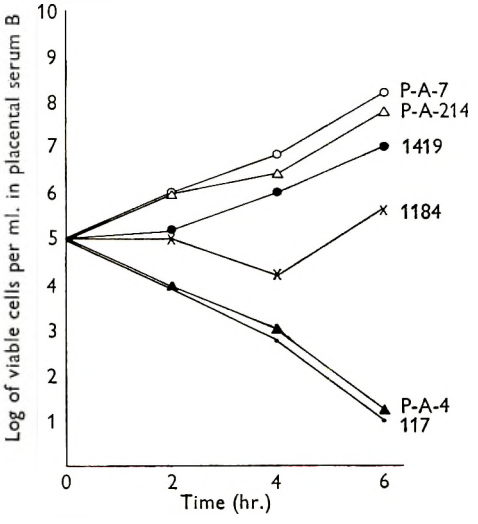

Fig. 4

Text-fig. 3. Growth curves of the 6 strains of *Ps. aeruginosa* in the placental serum A.

Text-fig. 4. Growth curves of the 6 strains of *Ps. aeruginosa* in the placental serum B.

DISCUSSION

The results of this study appear to indicate that the virulence of *Pseudomonas aeruginosa* depends on both the toxigenicity and the ability to grow in the serum of animals. Since the serum of many animals contains antibodies to various serological types of *Ps. aeruginosa*, a given strain of this species can show virulence to a particular animal only when its serum does not contain sufficient antibody to inhibit the growth of the organism, and then only if the organism is capable of producing various types of extracellular toxic substances. The fact that many samples of placental serum of man, and possibly those of various animals, contain antibodies in different degrees to different serological types of *Ps. aeruginosa* makes it very difficult, if not impossible, to describe the virulence of this species in general terms. Earlier observations on the antibody transfer in the human from mother to infant were concerned with diphtheria (Fischl & von Wunschheim, 1895) and tetanus (Polano, 1904). The subject of placental permeability to antibody has been reviewed by Doerr (1941). A reference to the virulence of *Ps. aeruginosa*, therefore, can be made with certainty only with regard to one particular strain of this species against one particular individual animal. This finding will explain the fact that some strains of *Ps. aeruginosa* isolated from severe cases of the infections, such as sepsis, do not

necessarily show more virulence in laboratory animals than those strains isolated from mild and trivial infections.

The relative resistance of man to infections with *Ps. aeruginosa* is probably due to both passive immunity acquired through the placenta and active immunity acquired through exposure to latent infections. Gaines & Landy (1955) have reported that antibody titres in man to the lipopolysaccharide of *Ps. aeruginosa* increased with age, and by 8–15 years practically all of those tested showed antibodies to this substance to some degree. It is interesting to note, however, that these antibodies were detected with the haemagglutination procedures. The same authors also mentioned that they were not able to demonstrate the presence of agglutinating antibody to bacterial cells in those sera containing haemagglutinating antibodies. Attempts to demonstrate incomplete antibody of the Coombs or blocking type also failed. Actually the failure to agglutinate the cells of *Ps. aeruginosa* by the sera containing antibody to the lipopolysaccharide can be explained by the fact that the cells of these organisms are usually covered by slime which could not be removed even by repeated washing with acid (Liu *et al.* 1961). Most of the bacterial suspensions used in the agglutination tests were prepared by exposure of these cells to formalin, phenol, alcohol, or heating which certainly will not remove these slimes on the surface of the cells of *Ps. aeruginosa*. It has been pointed out in a previous communication from this laboratory, (Liu *et al.* 1961) that it is the antibody to the slime, not the antibody to the lipopolysaccharide, that is responsible for both agglutination and protection. The slimes of *Ps. aeruginosa* have been shown to be a complex of large molecules containing carbohydrate, ribonucleic acid, deoxyribonucleic acid, and possibly others (Eagon, 1962). It did not appear to be as good an antigen as the lipopolysaccharide of the organism and, therefore, in the previous study mentioned above (Liu *et al.* 1961) an extensive use of Freund's adjuvant was made to produce an antibody to these substances. The poor antigenicity of slimes explains the fact that many sera of man or animals containing antibodies to the lipopolysaccharide of *Ps. aeruginosa* do not contain antibodies to the slimes.

Recently, Landy, Michael & Whitby (1962) described a method for measuring small amounts of antibody to Gram-negative bacilli which uses the bactericidal effect of the serum. These workers emphasized the fact that with this technique they were able to detect smaller amounts of antibody to enteric bacilli than by other serological tests. In the case of *Ps. aeruginosa*, however, such a technique would detect antibody to the slimes instead of those to the lipopolysaccharide. By testing the ability of *Ps. aeruginosa* to grow in undiluted sera of animals, we were actually performing quite a similar test to those described by Landy *et al.* (1962).

SUMMARY

The virulence of *Pseudomonas aeruginosa* appears to depend on both its ability to grow in the serum of animals and its ability to produce various types of extracellular toxins. No strain of *Ps. aeruginosa* lacking either of these qualifications was ever found to be virulent to animals.

The ability of various sera of animals to inhibit growth of *Ps. aeruginosa* appears to depend largely on their content of specific antibodies to each serological type of the surface antigens (the slime layer) and, therefore, susceptibility of animals to the infections of *Ps. aeruginosa*, even within one species, varies considerably from one individual to another.

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EXPLANATION OF PLATE 1

Lesions produced by 0·1 ml. of broth cultures of the 6 strains of *Ps. aeruginosa* in the skin of the rabbit no. 112. On the top, from left to right, are those produced by P-A-7 and P-A-214, both of which grew well in the serum of this rabbit and also produced considerable amounts of the extracellular toxins. In the middle, left side, is the lesion produced by P-A-4 which produced considerable amounts of the extracellular toxin *in vitro* but failed to grow in the serum of this rabbit. In the middle, right side, is the lesion produced by 1419 which grew well in this serum but failed to produce much extracellular toxins *in vitro*. On the bottom, from left to right, are those produced by 117 and 1184, neither of which grew well in this serum or produced much extracellular toxin. The last two strains hardly produced any change in the skin.

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A comparative study of susceptibility of primary monkey kidney cells, Hep 2 cells and HeLa cells to a variety of faecal viruses

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INTRODUCTION

During the routine virological and research investigations of this laboratory, many enteroviruses and adenoviruses were isolated on different tissue culture cell lines: the cell lines in use being primary monkey kidney cells and continuous HeLa and Hep 2 cells. Hsiung (1962) made a comparative study of the susceptibility of monkey kidney and human cells to enteroviruses using tissue culture passaged prototype strains while other workers (Archetti, Weston & Wenner, 1957; Stulberg, Page & Berman, 1958) had found that ECHO viruses grew less readily in permanent lines of human cells. It seemed useful to compare the primary isolation rates of the different viruses, including the vaccine strains of poliovirus, ECHO 6, ECHO 11 and other enteroviruses, and a variety of adenoviruses on the different cell lines used in this laboratory with particular reference to human malignant cells and primary monkey kidney cells.

MATERIALS AND METHODS

The HeLa cell line was grown in Hanks basic salts medium fortified with 0·5 % lactalbumen hydrolysate, 10 % human serum and 10 % calf serum and antibiotics (penicillin, streptomycin and nystatin). The Hep 2 cell line was originally obtained a number of years ago from the M.R.C. Virus Research Unit at Sheffield who in turn had obtained it from Dr R. Chanock; it was grown in the same medium as HeLa cells, except that there was no human serum present. Both these cell lines were maintained in medium 199 with 2 % calf serum added.

The primary cynomologous monkey kidney cells were grown in Hanks basic salt solution containing lactalbumen hydrolysate, 5 % horse serum and antibiotics and were maintained in serum-free medium 199. The specimens used in this study were the faeces of patients sent to the laboratory. Volumes of 0·2 and 0·1 ml. of a supernatant of a 10 % suspension of faeces were inoculated into two tubes. When the first sign of virus growth was observed, the number of days from the inoculation of the material was recorded. Cultures showing the toxic effect of faeces, or those cultures showing changes due to long maintenance, were passaged blindly. This was achieved by freezing and thawing them before they were inoculated into fresh tissue culture tubes. When the presence of a virus agent was suspected, confirma-

tion and identification was obtained by a neutralization test and in the case of adenovirus a complement fixation test as well. The techniques of virus isolation and neutralization were based on those originally described by Munro-Ashman, Gardner, Taylor & McDonald (1958) and Gardner, Knox, Court & Green (1962). All HeLa and Hep 2 cultures were kept a minimum of 28 days, while monkey kidney cells were kept about 14 days (see Table 1). When a child had recently been fed with oral poliovirus vaccine, it was assumed that the virus being excreted was a vaccine strain and no tests for virulence were performed.

RESULTS

During this study, vaccine strains of poliovirus, ECHO 6, ECHO 11, other enteroviruses and a variety of adenoviruses were isolated in the three different cell lines used. The results of isolation of vaccine strains of poliovirus in the cell lines are shown in Table 1.

Table 1. *Comparison of the three tissue culture lines for excretion of vaccine strains of poliovirus*

	Total no.	Positive monkey kidney	Positive Hep 2	Positive HeLa	Negative Hep 2 positive monkey kidney	Negative monkey kidney positive Hep 2
No. of poliovirus isolations	66	64 (97%)	64 (97%)	9 (14%)	2 (3%)	2 (3%)
Average time for isolating poliovirus	—	5 days	7 days	18 days	11 days	10 days
Average time of keeping negative cell lines	—	14 days	28 days	28 days	—	—

Of the specimens suspected of containing poliovirus, 97 % were positive both on monkey kidney and Hep 2 cells. The average time taken for isolating poliovirus on monkey kidney cells was 5 days and slightly longer on Hep 2 cells. In two specimens poliovirus was not isolated on Hep 2 cells but was recovered on monkey kidney cells and in a further two specimens virus was isolated only on Hep 2 cells. In nine specimens (14 %) only, were polioviruses isolated on HeLa cells and this was after prolonged incubation.

The results for the isolation of ECHO 6 virus on these cell lines are shown in Table 2.

On Hep 2 cells, ECHO 6 virus was isolated from 89 specimens, whereas only 42 % of these specimens showed positive results on monkey kidney cells. The average time taken for isolating ECHO 6 virus on Hep 2 cells was 9 days and slightly longer on monkey kidney cells. No isolations of ECHO 6 virus were ever made on HeLa cells even after 28 days incubation.

The results for the isolation of ECHO 11 virus on these cell lines are shown in Table 3.

ECHO 11 virus was isolated on Hep 2 from twenty-three specimens but only 52% of these specimens showed positive results when examined on monkey kidney cells. The average time for isolation of this virus on Hep 2 cells was 10 days and on monkey kidney cells slightly longer.

The results for the isolation of the various adenoviruses on these cell lines are given in Table 4.

Table 2. *Comparison of ECHO 6 virus isolations on the three cell lines*

	Total no.	Positive monkey kidney	Positive Hep 2	Positive HeLa
No. of ECHO 6 virus isolations	89	37 (42%)	89 (100%)	0
Average time for isolating ECHO 6 virus	—	11 days	9 days	—
Average time for keeping cell lines	—	14 days	28 days	28 days

Table 3. *Comparison of ECHO 11 isolations on the three cell lines*

	Total no.	Positive monkey kidney	Positive Hep 2	Positive HeLa
No. of isolations of ECHO 11 virus	23	12 (52%)	23 (100%)	0
Average time for isolating ECHO 11 virus	—	13 days	10 days	—
Average time for keeping cell lines	—	14 days	28 days	28 days

Table 4. *Comparison of adenovirus isolations on the three cell lines*

	Total no.	Positive monkey kidney	Positive Hep 2	Positive HeLa	Positive Hep 2 negative HeLa	Negative Hep 2 positive HeLa
No. of adenovirus isolations	25	9 (36%)	24 (96%)	22 (88%)	3 (14%)	1 (4%)
Average time for isolating adenoviruses	—	12 days	11 days	12 days	—	—
Average time for keeping cell lines	—	14 days	28 days	28 days	—	—

Table 5. *Comparison of other enterovirus isolations on the three cell lines*

Virus	Total isolated	Monkey kidney	Hep 2	HeLa
Coxsackie A9	2	2	—	—
Coxsackie B2	3	1	3	—
Coxsackie B3	3	2	3	—
Coxsackie B4	1	1	1	—
Coxsackie B6	2	1	2	—
ECHO 5	1	—	1	—
ECHO 9	1	1	—	—

Adenoviruses were isolated on Hep 2 cell lines from 96 % of suspected positive material and on the HeLa cells from 88 % of the same material. On three occasions HeLa cells showed complete negative results whereas an adenovirus was recovered on the Hep 2 cell line and in one specimen adenovirus was isolated on HeLa cells but not on Hep 2 cells. The average time for isolation of adenoviruses was 11 days on Hep 2 and 12 days on HeLa cells. Only 36 % of the specimens showed positive results on monkey kidney cells and the average time for isolation was again 12 days.

Table 5 shows a number of other enteroviruses which were isolated in the laboratory and which had been examined on all three tissues.

DISCUSSION

The results of this investigation have shed some light on to the susceptibility of a few of the common tissue cultures used in the laboratory for the isolation of faecal viruses.

The vaccine poliovirus strains grow equally well on monkey kidney cells and the Hep 2 cell line which were in use in this laboratory. It appears that where monkey kidney cells are not available, Hep 2 cell lines can be profitably used for the isolation of vaccine poliovirus strains in epidemiological problems without loss of sensitivity. On HeLa cell lines the vaccine strains did not grow well, only 14 % of these specimens showed positive results even after prolonged incubation. Virulent strains of polioviruses are known to grow as well on HeLa cells as on monkey kidney cells (Plotkin, Carp & Graham, 1962). Continuous cell lines have been used as markers for determining virulence of strains of polioviruses, viz. the M.S. marker (Kanda & Melnick, 1959), but in this case, Hep 2 cells are no use as a marker for avirulence.

Remarkably good results were obtained with Hep 2 cells for the isolation of both ECHO 6 and ECHO 11 virus when a comparison was made with monkey kidney cells, the Hep 2 cell line being far more sensitive for both these strains. The growth of ECHO 6 prototype strains on monkey kidney and Hep 2 cells is variable. Hsiung (1962) used a different cell line of Hep 2 (Sabin line) which was completely insusceptible to any of the ECHO prototype strains. He had, however, previously used a cell line, now lost due to bacterial contamination, which had been susceptible to a number of ECHO viruses. Hsiung also obtained a consistent positive result with rhesus monkey kidney cells, using ECHO 6 prototype strains. Other workers (Fukumi, Nishikawa & Mitzutani, 1958) found that certain virus strains related to ECHO 6 grew on HeLa cells but failed to grow on monkey kidney cells. The examination of prototype strains of ECHO viruses was purposely avoided in this study as they were all monkey kidney cultures and would have borne no resemblance to primary isolations.

Bell, Turner, Macdonald & Hamilton (1960) compared the susceptibility of different human cell lines (both HeLa cells and embryonic human tissues) to adenovirus type 3. They showed that human embryonic tissues were more susceptible than HeLa cells and compared the mean isolation time for their

respective isolations; it was found that embryonic tissues gave better results. Grayson, Loosli, Smith, McCarthy & Johnston (1958) demonstrated that different laboratory lines of HeLa cells varied in their sensitivity to adenovirus. The Hep 2 cell line used in this laboratory appeared to be slightly better than HeLa cells in their susceptibility to adenoviruses, though the line of HeLa cell in use was satisfactory (22 positive out of 25); the results also confirmed the observations of many that monkey kidney cells are not a good method for adenovirus isolation. In the small number of comparisons made with other enteroviruses there appear to be no significant differences in the susceptibility of Hep 2 cells and monkey kidney cells, with the possible exception of Coxsackie A 9.

The Committee on ECHO Viruses (1955) adopted the preferential susceptibility of monkey kidney cells as one of the criteria for the inclusion of a virus in the ECHO group. In view of our findings and those of others (Fukumi *et al.* 1958) either the various strains of viruses that have been isolated are not ECHO virus or the original definition of the ECHO group should be modified.

No hard and fast rules can be made as to which cell lines one should use in the laboratory. It has been shown that continuous cell lines, in this case Hep 2 cells, have a part to play in the isolation of enteroviruses. Laboratories where primary monkey kidney cells are difficult to obtain might do well to reconsider the host range of their continuous cell lines, in view of the experience of this laboratory.

SUMMARY

A comparative study of the susceptibility of monkey kidney, Hep 2 and HeLa cells to enteroviruses and adenoviruses is made. It seems that this Hep 2 cell line is as effective as the monkey kidney cells in their susceptibility to vaccine strains of polioviruses and far better than monkey kidney cells in their susceptibility to strains of ECHO 6 and ECHO 11 from clinical material. It is as effective as, or slightly better than, HeLa cells for the isolation of adenoviruses. It also compares favourably with monkey kidney cells for the isolation of other enteroviruses.

We are most grateful to Glaxo Laboratories Ltd. for the supplies of monkey kidney cells used in this investigation.

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Staphylococcus aureus type 83A as a cause of hospital cross-infection

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Numerous studies of cross-infection by *Staphylococcus aureus*, in which typing by bacteriophages has been used, have made it clear that the majority of outbreaks have been due to a relatively small number of phage types. Of these, type 80 appears to have been the most frequent; but other group I types, and certain types of group III have constantly recurred (Williams, 1959; Williams & Jevons, 1961).

Although another type, namely 83 A, has been referred to as causing a number of epidemics in surgical units (Williams, Blowers, Garrod & Shooter, 1960) and incidental references to infections caused by it have been made in other communications (e.g. Barber & Waterworth, 1962; Dickinson & Pride, 1962), no extended study of it as a cause of cross-infection appears to have been published.

It may be of value, therefore, to describe the part which it has played in staphylococcal cross-infection in a hospital group, over a period of $2\frac{1}{2}$ years. In this group, type 83A has shown a significant capacity to survive and spread in a hospital environment, and strains of it resistant to certain antibiotics have been isolated with a frequency not equalled by strains of other phage types.

MATERIALS AND METHODS

Period of study

A retrospective study has been made of the general incidence of this particular phage-type, over a period extending from October 1959 to March 1962.

Source of strains

The strains of *Staph. aureus* studied were isolated from clinical specimens during the routine work of a hospital laboratory. All were from in-patients of four main hospitals of a group. These were: a general hospital, acute medical and surgical; a fever hospital, also containing wards for acute medical cases from which most, if not all, of the staphylococci from this hospital were derived; a chest hospital; and an ex-poor-law hospital which now houses orthopaedic, geriatric and maternity units.

Only those strains which could reasonably be supposed to be playing a pathogenic role are included in the survey; this criterion has been difficult to apply in some cases, notably in the case of strains isolated from sputum.

Repeated isolations from a single patient have been excluded, except where the

later isolation has come from a different lesion from the earlier one, or has been of a different phage type.

All strains were, by definition, coagulase-positive. The terms 'staphylococcus' and 'staphylococci', when used without qualification in this paper, should be taken to mean coagulase-positive *Staph. aureus*.

Drug sensitivity tests

These were done routinely by a disk-plate method, tube dilution tests being done in a few cases where indicated.

Phage typing

During the relevant period, all coagulase-positive staphylococci that were isolated, and which were found to be resistant to one or more of the antibiotics used in the routine sensitivity test, were subcultured to agar slopes and stored for phage-typing. Typing was done in batches, the usual interval being 7–14 days.

The methods of typing used were those described by Williams (Anderson & Williams, 1956), and Blair & Williams (1961). The standard, internationally agreed set of 21 phages was used, plus phage 83A which throughout the period of the survey did not form part of that set.

RESULTS

Type 83A: powers of survival and spread

The total number of strains of *Staph. aureus* isolated during the period, which were likely to be acting as pathogens and which were resistant to more than one antibiotic, was 1131. Of these, 776 were resistant to tetracycline.

Since it has been found here, as elsewhere, that tetracycline-resistance in hospital staphylococci is a reasonably accurate index of their capacity to produce cross-infection, such strains only have been considered in Table 1, which has been designed to show how type 83A has, throughout the $2\frac{1}{2}$-year period, continued to produce cases of cross-infection in the four hospitals.

So far as 83A itself is concerned, strains isolated during the period were almost invariably resistant to tetracycline (sensitive, 17; resistant, 263 strains). In addition, tetracycline-resistance, both in 83A strains and those of other phage-types was almost uniformly associated with resistance to penicillin and streptomycin.

In Table 1 the numbers of strains of tetracycline-resistant staphylococci isolated every quarter have been divided into those of type 83A, and those belonging to other phage-types; although, for convenience, the latter heading has been made to include also any untypable strains.

Among 'other types' those of group I greatly predominated, 52/52A/80 or 52/52A/80/81 being the commonest, followed by type 80 or 80/81. Group III strains, excluding 83A, followed in order of frequency, with a smaller number of strains lysed by phages of both groups I and III. Untypable strains numbered 89, or 11·3% of the total.

It will be seen that over the whole period some 34% of tetracycline-resistant strains were type 83A. It will also be seen that there was a rise in the number of

such strains in the first half of 1960, and a fall in the middle of 1961, but that, apart from these fluctuations, type 83A has pretty consistently constituted about one-third of the tetracycline-resistant strains isolated. It may, therefore, be reasonably supposed to have been responsible for something like one-third of the staphylococcal cross-infection occurring during this time.

Table 1. *Numbers of tetracycline-resistant* Staph. aureus, *isolated every quarter, October* 1959 *to March* 1962, *in four hospitals: showing the incidence of type* 83*A as compared with other types*

Hospital...	A		B		C		D		Totals		
Phage-types...	83A	Other types	83A	Other types	83A	Other types	83A	Other types	83A	Other types	83A (%)
Period											
1959											
Oct.–Dec.	7	17	4	10	14	14	1	12	26	53	32·9
1960											
Jan.–Mar.	10	21	7	5	9	10	4	6	30	42	41·7
Apr.–June	14	19	4	4	12	15	6	12	36	50	41·9
July–Sept.	1	10	1	4	7	7	7	10	16	31	34·0
Oct.–Dec.	3	10	1	9	11	8	6	7	21	34	38·2
1961											
Jan.–Mar.	4	26	1	14	10	6	15	10	30	56	34·9
Apr.–June	5	22	2	8	4	10	10	15	21	55	27·6
July–Sept.	4	23	3	11	3	7	5	16	15	57	20·8
Oct.–Dec.	7	24	7	9	8	12	7	15	29	60	32·6
1962											
Jan.–Mar.	11	23	6	3	17	20	5	29	39	75	34·2
Totals	66	195	36	77	95	109	66	132	263	513	33·9
83A (%)	25·3	—	31·9	—	46·6	—	33·6	—	33·9	—	—

Hospital A = general; B = fever and acute medical; C = chest; D = Orthopaedic, geriatric and maternity.

In the chest hospital about half the tetracycline-resistant strains belonged to type 83A, in the general hospital about one-quarter and in the other two hospitals about one-third. The high incidence of one particular type in four hospitals, the nearest of which are some miles apart, and which deal with a wide variety of clinical material, is a striking feature.

Type 83*A: resistance to chloramphenicol, erythromycin and novobiocin*

Staphylococci were not phage-typed unless resistant to one or more of the antibiotics routinely tested. These were: penicillin, streptomycin, chloramphenicol, tetracycline, erythromycin and novobiocin.

Where there was resistance to more than one antibiotic the commonest pattern was: resistant to penicillin, streptomycin, tetracycline; sensitive to chloramphenicol erythromycin, novobiocin. Resistance to any of the last three antibiotics was rare, but, during the period of study, it became apparent that if a strain did show

resistance to any of these it was likely to be of phage type 83A. Table 2 expresses this quantitatively.

It will be seen that the percentage of all strains resistant to chloramphenicol was 7·0, whereas the percentage of 83A strains thus resistant was 11·8. This is significantly more than occurs with strains of other groups, except group II, and here the number of strains involved is so small that their significance is doubtful. Of untypable strains, however, 8·8 % were resistant.

The resistance rate to erythromycin is more striking, 29·6 % of 83A trains being resistant, while the group with the next largest percentage—group III, excluding 83A—provides only 4·9 % of resistant strains.

Table 2. *Phage types of strains of* Staph. aureus *resistant to chloramphenicol, erythromycin and novobiocin*

Type or group	Total no. of strains	No. of strains resistant to					
		Chloramphenicol		Erythromycin		Novobiocin	
		Total	%	Total	%	Total	%
83A	280	33	11·8	83	29·6	25	8·9
Group I	405	17	4·2	9	2·2	12	3·0
Group II	26	3	11·5	1	3·8	0	—
Group III other than 83A	203	9	4·4	10	4·9	0	—
Other types	46	2	4·3	2	4·3	0	—
Untypable	171	15	8·8	7	4·1	1	0·6
Totals	1131	79	7·0	112	9·9	38	3·4

So far as noboviocin-resistance is concerned, the small number of strains makes their significance more doubtful. However, of the 38 resistant strains, 25 belonged to phage-type 83A.

Type 83A: pathogenicity

To gauge the significance of 83A strains it is necessary to know not only the total number of isolations, but also something of the lesions which they caused and the severity of these.

The latter, in a retrospective survey, can be assessed only in the most general terms; but some idea of the nature of the infections can be gained from the clinical sources of the strains, and these are shown in Table 3.

This table deals with the 776 tetracycline-resistant strains already considered in Table 1: its value as evidence of infection varies with the clinical source from which such a strain was derived.

Strains from operation wounds may be taken to show true secondary infection in practically all cases, and provide an index of the capacity of type 83A to produce such infection. The second category of the table—'abscesses, boils, etc.'—covers a wide variety of infections of the skin and subcutaneous tissue, but here again the staphylococci isolated may almost invariably be taken to be causative.

The case is otherwise with strains isolated from sputum; and although a number were not included in the series as not significant, because of scanty growth or other

reasons, an unknown number of 'carrier strains' must still remain. On the other hand, among ample evidence to show that type 83A strains are capable of causing severe chest infection, is the fact that such strains were isolated post mortem from the lung on nine occasions. In five of these cases it appeared likely that the staphylococcus was the cause, or major contributory cause, of death.

There is no doubt about the pathogenic role of the urinary strains; and strains from faeces were all from cases which appeared to be true staphylococcal entero-coliti Of the eighteen cases of entero-colitis, fourteen were caused by type 83A with one death.

Table 3. *Nature of infections caused by tetracycline-resistant strains of type* 83*A and those of other types*

Phage-type	83A	Other types	83A (%)
Clinical source of strains			
Operation wounds	35	68	33·9
Abscess, boils, etc.	31	94	24·8
Sputum	109	224	32·7
Eye	16	27	37·2
Ear, nose, throat	13	14	48·1
Urine	17	35	32·7
Faeces	14	4	77·8
Other sources	28	47	37·3
Totals	263	513	33·9

'Other sources' comprises a wide variety of infections, but a case of meningitis caused by type 83A is worth mentioning, as the only case of staphylococcal meningitis during the period. This was a fatal infection in a woman aged 74.

DISCUSSION

This survey is concerned only with the incidence of type 83A in the four hospitals during the period studied. No attempt has been made to describe individual outbreaks, or the investigation of these and the measures which were taken to combat them.

In spite of such measures it will be seen that staphylococcal cross-infection continued throughout the period, and that type 83A was responsible for about one-third of it.

The objects of this communication are, first, to draw attention to these powers of survival and spread of this type, and to suggest that its appearance in an environment where it has not previously been found should be taken seriously; and, secondly to indicate the frequency with which strains resistant to chloramphenicol, erythromycin and novobiocin may be encountered.

Since this work started, however, erythromycin and novobiocin have lost their pride of place in the treatment of multiple-resistant staphylococcal infections, to be supplanted by methicillin and cloxacillin. It has been of interest, therefore, particularly in view of their resistance to other antibiotics, to test strains of 83A against methicillin; and 214 such strains were thus tested, between September

1960 and May 1962, using a disk-plate method with 10 μg. of methicillin in the disk. All strains were isolated from clinical specimens, but 32 were considered not to be acting as pathogens. Repeat isolations from the same patient were excluded. None of the strains tested by this method showed resistance.

This is encouraging, but it should be recorded that Barber & Waterworth (1962) have reported that of 4017 strains of staphylococci, 1078 being from infected patients, tested against methicillin, 88 strains showed some degree of resistance, and of these 88 strains, 12 belonged to phage-type 83A. Barber & Waterworth used a ditch-plate method of testing, and it seems possible that this might pick up minor degrees of resistance more frequently than the antibiotic disk.

It is, incidentally, of some interest, in view of our finding concerning erythromycin-resistance among 83A strains, that the 12 methicillin-resistant 83A strains isolated by Barber & Waterworth were all resistant also to erythromycin; whereas their remaining 76 methicillin-resistant strains of other phage types were all erythromycin-sensitive.

The prominence of 83A strains as a cause of cross infection in four widely-separated hospitals, over a period of more than $2\frac{1}{2}$ years; the power of these strains, on occasion, to produce severe and sometimes fatal infection; the number of strains shown to be resistant to chloramphenicol, erythromycin or novobiocin, in addition to other antibiotics; and the demonstration, elsewhere, that 83A is one of the phage types of which there are very few at present, which may show resistance to methicillin, form a combination of characters which suggests that, even in comparison with other well-known epidemic types, 83A may be a particularly noteworthy cause of hospital cross-infection.

SUMMARY

1. A retrospective survey has been made to determine the amount of cross-infection caused by *Staphylococcus aureus* of one particular phage-type, 83A, in four main hospitals of a provincial hospitals group, over a period of $2\frac{1}{2}$ years.

2. The total number of strains considered was 1131. Of these, 776 were resistant to tetracycline and therefore the most likely to be of significance in cross-infection. Of these 776 strains, about one-third belonged to phage-type 83A. In the hospital where 83A strains were most prevalent, they formed about one-half of the tetracycline-resistant strains; in that where they were least prevalent, about one-quarter.

The clinical sources of the tetracycline-resistant strains are described.

3. It was found that type 83A strains were the most likely to show resistance to erythromycin, chloramphenicol or novobiocin.

Of a series of 214 type 83A strains sensitivity tested against methicillin, using a disk-plate method, none was found to be resistant.

My thanks are due to Prof. R. E. O. Williams, St Mary's Hospital Medical School, for his helpful criticism of this paper and, in his previous capacity as Director of the Staphylococcus Reference Laboratory, Central Public Health Laboratory, Colindale, to him and his staff for supplying me with typing phages,

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Liberation of organisms from contaminated textiles

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INTRODUCTION

In an earlier study (Rubbo, Stratford & Dixson, 1962) it was shown that hospital blankets, artificially contaminated with a marker organism, *Staphylococcus citreus*, liberated more organisms in the ward environment when they were covered with laundry-clean counterpanes than when they were not. This curious 'counterpane effect' appeared to be due to the removal of bacteria-carrying particles from the contaminated surface by friction with the overlying counterpane.

In the present work this 'counterpane effect' has been further investigated. Apart from examining the mechanisms of liberation of organisms from contaminated textiles it was also possible to evaluate the degree of dispersion of organisms from different textiles carrying the same bacterial loading. The results support the view that friction between contaminated surfaces contributes heavily to the airborne dispersion of organisms and that cotton fabrics tend to yield a higher number of airborne organisms on agitation than woollen materials.

METHODS

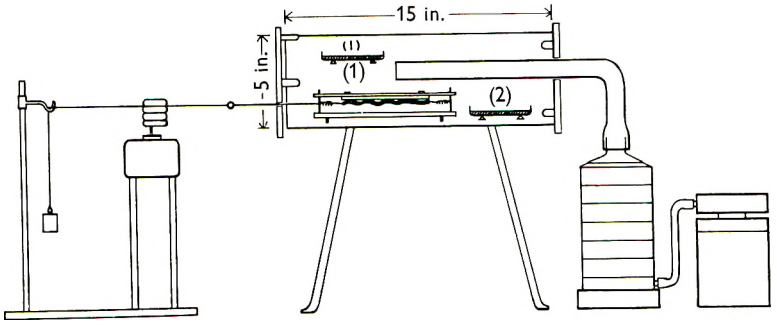
The general procedure used in this study was to contaminate a known area of sterile textile and then to determine the yield of airborne organisms on agitation in a specially designed apparatus (see Text-fig. 1 and Pl. 1 B). The degree of surface contamination was measured by the contact plate method (Rubbo & Dixson, 1960) and airborne counts were made with an Andersen sampler (Andersen, 1958). In all cases Difco brain heart infusion agar containing 8 % sodium chloride was used. The relevant details of this general procedure are as follows:

Textiles. New and used textiles consisting of pure wool, wool-cotton mixture, acrilan and cotton were tested. Each was cut into $6\frac{1}{4}$ in. $\times$ $3\frac{1}{4}$ in. samples and stapled at the corners. In addition, circles $3\frac{1}{2}$ in. in diameter were prepared for contact plating. All were dispensed in paper envelopes having cellophane-covered windows measuring $4\frac{1}{2}$ in. $\times$ 2 in. or 3 in. circles. The envelopes containing the textile samples were sterilized in 0·15 % propylene oxide vapour at 56° C. for 24 hr. in a sealed container in an atmosphere of 70 % relative humidity. The sterile samples were stored at room temperature for at least 48 hr. before use.

Contamination of textiles. The textiles were artificially contaminated by mounting the envelope on a copper frame, removing the cellophane window and placing the frame in a predetermined position in a desiccator jar (Pl. 1 A). A suspension of

Staph. citreus, prepared by diluting a 24 hr. broth culture 1 in 10 in saline, was atomized into the sealed jar containing the exposed textiles. The time allowed for contamination was 7 min. consisting of 2 min. atomization period and a 5 min. fall-out time. The air pressure was regulated by a reducing valve to 15 lb. per sq.in.

Natural contamination of textiles in various wards was done by placing the envelopes, with their cellophane windows removed, on perforated metal trays mounted 3 and 6 ft. above floor level. The exposures were made in four bed surgical wards for 2 to 3 days.



Text-fig. 1. Drawing of apparatus illustrated in Pl. 1B showing position of settle plates (1) and (2).

Liberation of organisms from contaminated textiles. This was done under constant conditions using the apparatus shown in fig. 1 above and Pl. 1B. The method of using this apparatus might be briefly mentioned. The contaminated textile, mounted on springs (Pl. 2A), was shaken by the rotation of a spindle mounted off-centre on a small electric motor. The shaking was standardized to 1500 $\frac{1}{2}$ in. oscillations per minute for 5 min. The shaking took place freely or in contact with a metal plate (aluminium) mounted above the textile or in contact with a second textile fixed to the metal plate (Pl. 2B). During the 5 min. period of agitation air was passed through the chamber into an Andersen sampler. The efficiency of this method of sampling airborne organisms was checked by exposing two settle plates in the chamber in every experiment. All air samplings recorded are for 5 cu.ft. of air collected at the rate of 1 cu.ft./min.

A typical experiment proceeded as follows. The interior of the collecting chamber and the removable Perspex end-plates were swabbed with 70 % alcohol. The contaminated textile was mounted on the springs of the carrier and a paper clip attached to one end (Pl. 2A). Depending on the experiment, the metal plate, with or without the textile cover, was fixed to the carrier (Pl. 2B) so that contact between the spring-mounted textile and the plate was intermittently established during shaking. The carrier was then inserted in the chamber in a fixed position and two settle plates were placed in positions indicated in Text-fig. 1. The Perspex end-plates were mounted at either end of the collecting chamber; an air space, about $\frac{1}{4}$ in., was left at the end-plate nearest the shaker. Through a hole in this plate a wire loop was connected to the paper clip attached to the textile (Pl. 2B).

Through the other end-plate a glass collecting tube (1 in. diameter) was inserted and connected to the Andersen sampler (Pl. 1 B). Shaking the textile and air sampling were started simultaneously and continued for 5 min. The Andersen salt agar plates and the settle plates were then incubated for 48 hr. at 37° C. and counted. The Andersen plate counts were adjusted as recommended. The shaken fabric was sampled by the contact plate method and an unshaken control textile, similarly contaminated, was also sampled by contact plating.

RESULTS

In Table 1 the number of airborne organisms released on shaking artificially contaminated textiles is shown. It can be clearly seen that both woollen and cotton blankets release significantly more organisms when they are shaken in intermittent contact with a second textile, e.g. cotton sheet. On the other hand, shaking in contact with a smooth metal, such as aluminium, did not produce a higher yield of airborne particles from either material.

Table 1. *Release of airborne organisms on shaking artificially contaminated textiles*

Contamination: *Staph. citreus* (see text)

Textile	Condition of shaking (see text)	No. of expts.	Andersen sampler counts (av.) Stage 1	2	3	4	5	6	Total count
Woollen blanket	No contact	4	0	0	1	7	11	0	19
(75 % wool 25 % cotton)	Metal contact	4	2	2	2	6	4	0	16
	Sheet contact	4	1	2	7	29	22	9	70
Cotton blanket	No contact	4	1	1	1	12	18	1	34
(Terry towel)	Metal contact	4	1	1	2	14	23	1	42
	Sheet contact	4	1	4	7	62	56	0	130

Confirmation of these results was obtained by determining the extent of removal of organisms by contact plating textiles before and after shaking. Contact plates were made on separate samples of textiles contaminated at the same time as the sample to be tested, which, after shaking, was also contact plated. In every case the removal of organisms from the contaminated surface was significantly higher when shaken in contact with a second textile, Table 2. For example, the drop in surface count when a woollen blanket was shaken alone was 52 % but when shaken in contact with a cotton sheet the removal was 98 %. Similar differences are seen with other textiles as shown in Table 2.

By using the Andersen sampler it was possible to determine approximately the particle size distribution of the atomized suspension used for contaminating the textiles and the size of bacteria-carrying particles released when the textiles were shaken. The results are shown in Table 3. It is evident that the airborne organisms liberated from the textiles were of the same particle size as those which were used in contaminating the textiles.

Settle plate counts in all the above experiments and those reported later were negligible. Usually the plates were sterile or if growth occurred never more than two colonies were recovered. In short, the Andersen plate counts truly represent all the airborne organisms recoverable in the collecting chamber.

The results obtained with textiles contaminated by exposure to ward air reflect the same pattern as was found with the artificially contaminated samples, namely, an increased yield of airborne organisms when shaken in contact with a second textile (Table 4).

Table 2. *Removal of surface organisms on shaking artificially contaminated textiles*

Contamination: *Staph. citreus*

Textile	Condition of shaking	No. of expts.	Contact plate counts (av.) Before shaking	Contact plate counts (av.) After shaking	Removal of surface organisms (%)
Woollen blanket (75 % wool 25 % cotton)	No contact	7	220	105	52
	Sheet contact	8	183	7	98
Cotton blanket (Terry towel)	No contact	6	211	84	60
	Sheet contact	5	276	21	93
Acrilan blanket	No contact	4	399	230	42
	Sheet contact	7	282	7	98

Table 3. *Particle size distribution of airborne organisms released on shaking artificially contaminated textiles*

Contamination: *Staph. citreus*

Textile	Condition of shaking	No. of expts.	Andersen sampler count (av.) total > 6 μ (1 2 3)	Andersen sampler count (av.) total < 3·5 μ (4 5 6)	Particle size distribution (%) > 6 μ	Particle size distribution (%) < 3·5 μ
Woollen blanket (100 % wool)	No contact	5	2	24	8	92
	Sheet contact	5	6	42	11	89
Woollen blanket (75 % wool 25 % cotton)	No contact	4	1	18	6	94
	Sheet contact	4	10	60	14	86
Cotton blanket (Terry towel)	No contact	4	3	31	11	89
	Sheet contact	4	12	118	8	92
Staph. citreus (for comparison)	10^{-3} dilution atomized in 10 % broth	8	20 3 17	202 261 6	8	92

It is interesting to note that the natural contaminants in the ward air were delivered to the textiles on much larger particles than those used for artificial contamination (see Tables 3 and 4). When the particle size distribution of airborne organisms arising from the same textiles is considered, it is evident that textiles

shed their contaminant flora on much the same size particles as those which were responsible for the contamination.

Table 5 confirms the findings listed in Table 4 by demonstrating that the most effective removal of surface organisms is seen when a textile is shaken in contact with another fabric.

Table 4. *Release of airborne organisms on shaking naturally contaminated textiles*

Contamination: exposure to ward air for 2–3 days

Textile	Condition of shaking	No. of expts.	Andersen sampler counts (av.) Stage > 6 μ 1	2	3	< 3·5 μ 4	5	6	Total count	Particle size distribution 90 particles (%) > 6 μ	< 3·5 μ
Woollen blanket (100 % wool)	No contact	5		8			1		9	89	11
	Sheet contact	5		21			1		22	95	5
Woollen blanket (75 % wool 25 % cotton)	No contact	4		6			1		7	86	14
	Sheet contact	4		11			3		14	79	21
Cotton blanket (Terry towel)	No contact	4		16			1		17	94	6
	Sheet contact	4		20			7		23	87	13
Acrilan blanket	No contact	3		4			1		5	80	20
	Sheet contact	4		10			1		11	91	9
Ward air for comparison		9	43	30	21	12	21	2	129	73	27

Table 5. *Removal of surface organisms on shaking naturally contaminated textiles*

Contamination: exposure to ward air for 2–3 days.

Textile	Condition of shaking	No. of expts.	Contact plate counts (av.) Before shaking	After shaking	Removal of surface organisms (%)
Woollen blanket (75 % wool 25 % cotton)	No contact	4	63	27	58
	Sheet contact	4	63	4	92
Cotton blanket (Terry towel)	No contact	4	70	21	70
	Sheet contact	4	70	3	96
Acrilan blanket	No contact	3	34	41	0
	Sheet contact	4	30	1	97

DISCUSSION

It is generally considered that agitation of a textile, e.g. a blanket, provides the principal mechanism for the airborne dispersion of its bacterial flora. The present study, and the one which preceded it (Rubbo *et al.* 1962), show that the yield of airborne organisms from a textile is greatly increased if the agitation is carried out in contact with a second textile (Tables 1 and 4). It would appear that the frictional contact between the two textile surfaces is a necessary prerequisite for

the maximum liberation of organisms from these surfaces. The increase in aerial contamination due to friction is at least a twofold one and similarly the increased removal of organisms from a contaminated surface is of the same order. In contrast, shaking in contact with a smooth metal surface does not increase this yield of airborne organisms (Table 1).

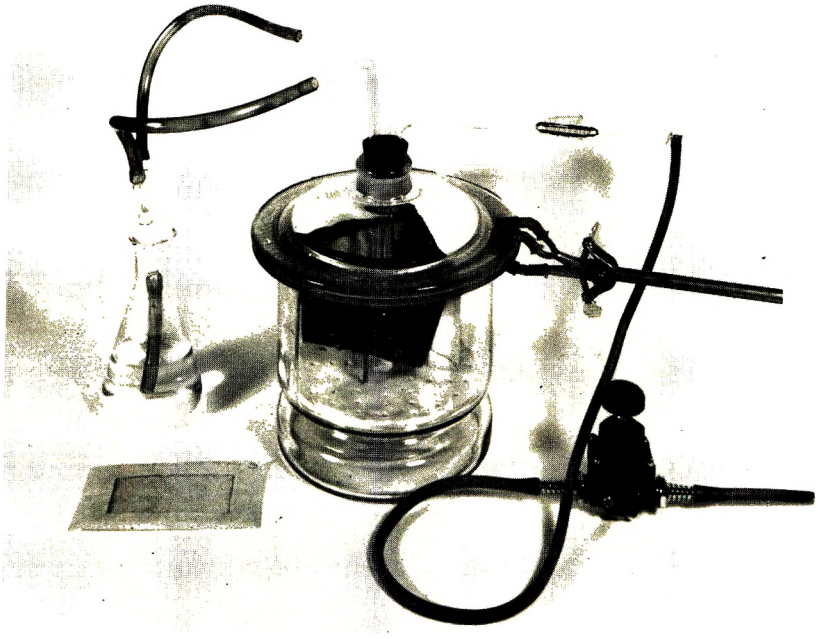
These findings confirm previous conclusions which were drawn from the study of the spread of a marker organism in a hospital ward. The 'counterpane effect', as we previously termed it, described the increased dispersion of organisms when a contaminated blanket is covered by a laundry-clean counterpane (or quilt). In the light of these two parallel studies one can assume that one way of reducing aerial broadcast of organisms from contaminated blankets is to refrain from covering them with counterpanes and to expose only one side of the blanket during use.

In regard to the yield of airborne organisms produced by woollen and cotton blankets, it will be seen (Table 1) that when the two types of blanket are artificially contaminated and tested under the same conditions the cotton blankets always produce more airborne particles than the woollen types. Naturally contaminated woollen and cotton blankets yielded about the same number of organisms when shaken in contact with a sheet but shaken alone the cotton blankets gave higher counts (Table 4). Thus, from the point of view of airborne spread of infectious particles, one cannot claim any hygienic advantage in replacing woollen blankets with cotton ones.

This study provides no direct information on the nature of bacteria-carrying particles released from textiles. We have been able to show that there was no significant shift in the particle size distribution between airborne organisms contaminating a textile and those released when it was shaken. Since most bacteria found in the air of hospital wards are carried on particles about 10–20 μ in size, their origin is of theoretical as well as practical interest (Rubbo, 1963). We have suggested, on indirect evidence, that infectious airborne particles consist of minute cellulose fibres originating from dressings, sheets, pillow slips, etc., and contaminated by discharges and secretions of their users (Rubbo, Pressley, Stratford & Dixson, 1960). More recently Davies & Noble (1962) have suggested that airborne organisms dispersed from carriers and from their bedding are associated with desquamated skin. It would appear that the airborne particles released by textiles, for which we have used the term fibre nuclei, consist of both skin scales and fragmented cellulose fibres, the former arising from the clothing of skin carriers and the latter from contaminated dressings of patients. Whatever may be their true nature it is clear from the present study that the aerial dissemination of these particles will be determined to a large extent by the degree of friction between the contaminated textile surfaces from which they arise.

SUMMARY

By using a specially designed apparatus it was possible to study the yield of airborne organisms when various hospital textiles, artificially and naturally contaminated, were shaken under reproducible conditions. The results indicated that contaminant organisms are most effectively liberated when a textile is shaken in



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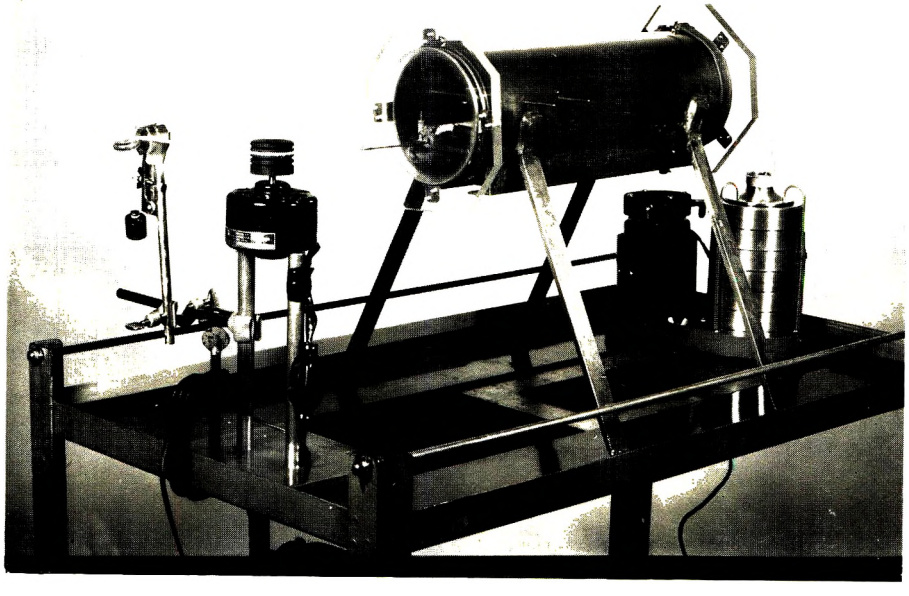

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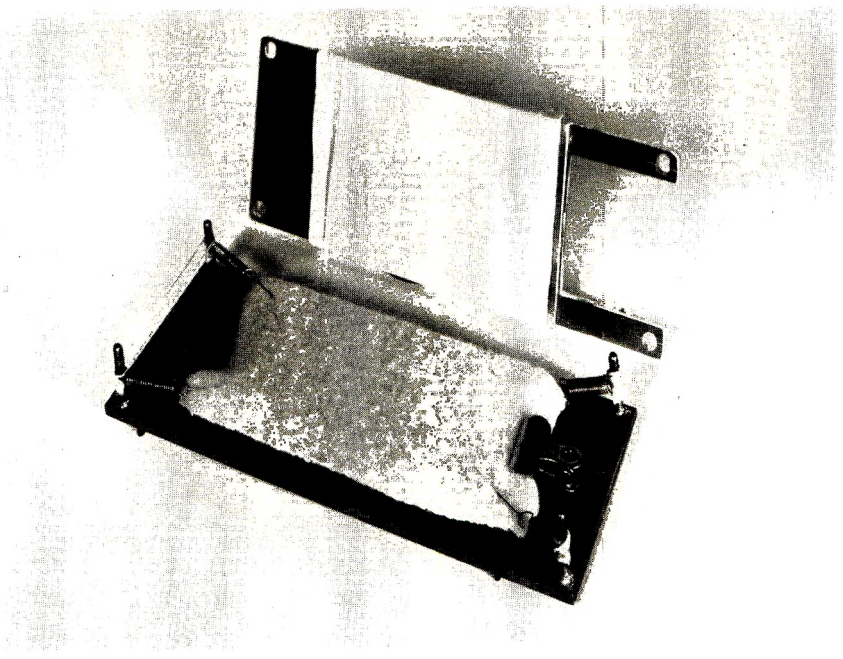

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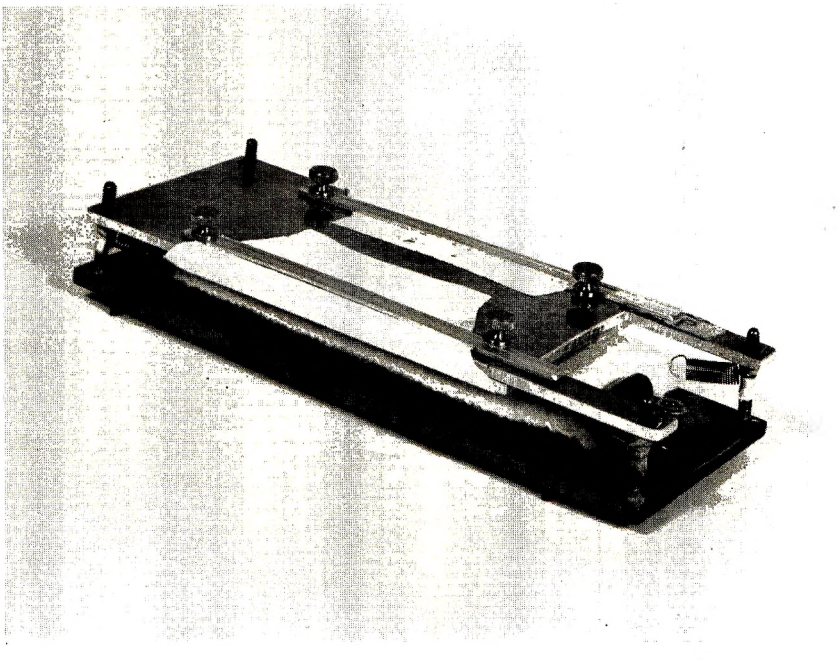

(B)

contact with a second fabric. The yield of airborne organisms was approximately doubled under the conditions used. Similarly, a 95% removal of surface contaminants was also demonstrated by shaking in contact with a second textile, compared to 52% removal when the textile was shaken alone.

These findings confirm those described in an earlier study of the spread of airborne organisms in a ward environment. It is suggested that this increase in aerial spread of infectious particles, previously referred to as a 'counterpane effect', is due to friction between the contaminated textile and its covering material.

Other points which emerged in the course of this work were the demonstration that cotton blankets tended to yield higher airborne counts than woollen ones and that the size distribution of bacterial particles dispersed by textiles is the same as that of the particles which contaminate the textiles.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

A. Method of artificially contaminating a textile with atomized suspension of *Staph. citreus*.
B. The apparatus for collecting airborne organisms during shaking.

PLATE 2

A. Method of mounting contaminated textile on carrier and method of fixing second textile.
B. The two textiles mounted in position on the carrier.

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The use of gamma radiation for the elimination of salmonellae from various foods

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INTRODUCTION

A wide variety of foods have been implicated as sources of salmonellae. As pointed out by Hobbs (1962) in a paper summarizing the current position, certain imported foods are heavily contaminated. Present methods employed for the elimination of these pathogens, when the hygiene of production has failed, are based on the use of heat treatment, but gamma radiation is proposed as an alternative process offering important advantages for some products. The radiation is penetrating; it can be applied without removing the food from its container, and the temperature rise during treatment is negligible; hence its particular applicability to frozen items such as whole egg and meats.

The usefulness of gamma radiation for the treatment of frozen whole egg was recognized earlier by Brooks, Hannan & Hobbs (1959), and more detailed bacteriological studies on this product are reported in this paper. Evidence is also presented on the use of the process for the elimination of salmonellae from frozen horse-meat, desiccated coconut, and bone meal. The practical dose requirement is estimated from the results of experiments on artificially or naturally contaminated material, and the inactivation factors realized are calculated from dose/survival curves prepared for different serotypes in each product. For comparative purposes the results are also given for the radiation resistance of salmonellae in a buffer solution under various environmental conditions.

MATERIALS AND METHODS

Dose/survival curve experiments

Materials

Home-produced frozen whole egg; frozen boneless horse-meat as imported in blocks; desiccated coconut purchased locally, packaged as for retail sale. Bone meal imported for use as fertilizer.

M/15 phosphate buffer of neutral pH.

Salmonella *serotypes*

Five serotypes were used during the course of these studies: *Salmonella typhi-murium* phage-type 2c (14), *S. senftenberg*, N.C.T.C. 9959 (775 w), *S. paratyphi* B phage-type Beccles var. 3, *S. gallinarum* and *S. meleagridis*. *S. typhi-murium* was used in all the experiments because it has been isolated from each of the types of food under investigation and was detected in 64 % of food-poisoning incidents in human beings in 1961 (see Report, 1962*a*). The heat-resistant strain of *S. senftenberg*, N.C.T.C. 9959 (775 w), was chosen in order to check any possible relationship with radiation resistance.

Inoculation and irradiation

Nutrient agar slope cultures grown for 18–24 hr. were used throughout. Before inoculation each of the foods studied, with the exception of coconut, was irradiated with a dose of 1 Mrad. (in the frozen state for meat and whole egg) to remove organisms originally present, thus simplifying the recovery and counting of salmonellae. General observations on the growth characteristics of salmonellae in the irradiated food showed that such treatment did not influence the effectiveness of the food as a growth medium. Bacteriological examination of the desiccated coconut showed that it was only slightly contaminated and therefore pre-treatment by irradiation was unnecessary.

The different serotypes were studied separately in each of the following media.

(*a*) *Whole egg*. The slope culture was washed off with 10 ml. buffer, and 0·5 ml. of this suspension was added to 200 ml. of liquid whole egg and mixed. After incubation at 37° C. for 3 days the colony count of each serotype used was approximately 10^8/ml. Ten ml. quantities of infected egg were placed in universal containers for irradiation in the liquid state and 30 ml. quantities were placed in aluminium cans and frozen rapidly at − 17° C. for irradiation in the frozen state. The cans were maintained frozen during irradiation by surrounding them with solid CO_2 in an outer vessel. The cans were subsequently placed in water at 37° C. until thawed.

(*b*) *Frozen horse-meat*. The meat was thawed and cut into 20 g. samples which were placed individually in aluminium cans with screw caps. The slope culture was washed off with 5 ml. of blood exudate from the thawed meat, and 0·3 ml. of this suspension was inoculated into the centre of each meat sample. After overnight (16 hr.) incubation at 37° C. and then rapid re-freezing at − 17° C., the colony counts for salmonellae were approximately 5×10^8/g. The samples were maintained frozen during irradiation as described for whole egg, and thawed in a similar manner after treatment. Each sample was macerated with 100 ml. sterile distilled water in an M.S.E. homogenizer, and from this suspension 1 ml. portions were withdrawn to make suitable dilutions.

(*c*) *Desiccated coconut*. The slope culture was washed off with 10 ml. of buffer and 0·5 ml. of the suspension (10^9 orgs./ml.) was added drop by drop to each 3 g. sample of coconut in a sterile Petri dish. The coconut was dried at 37° C. for $1\frac{1}{2}$ hr. with the lids of the dishes raised. The initial colony count was approximately

10^8/g. The dried samples were transferred to universal containers for irradiation. Buffer solution, 20 ml., was added after irradiation and the containers were shaken vigorously for 5 min., after which 0·5 ml. portions were withdrawn for dilution and plate count.

(*d*) *Bone meal.* Concentrated suspensions of salmonellae were prepared by washing off cultures from four nutrient agar slopes with buffer, centrifuging and resuspending in 5 ml. of buffer. The final concentration was 10^{10} orgs./ml., and 1 ml. was added drop by drop to 5 g. samples of bone meal in Petri dishes as for the coconut. After drying at 45° C. for 1 hr., irradiation was carried out in the same way. The initial plate count was 10^7/g. After irradiation, 10 ml. of phosphate buffer were added and the containers shaken vigorously for 5 min. before withdrawal of 0·5 ml. quantities for dilution and plating.

(*e*) *Buffer suspensions.* The slope was washed with 10 ml. buffer, centrifuged for 30 min. and re-suspended in 10 ml. of buffer to give a final concentration of 10^9 orgs./ml. For non-aerated conditions the suspension was irradiated in a universal container. To obtain aeration conditions, air was bubbled through the suspension in suitable glass vessels throughout the irradiation period. Anoxic conditions were obtained by bubbling 'oxygen-free' nitrogen through the suspension for 6 min. before and throughout irradiation. For irradiation in the frozen state the suspension was held for 1 hr. at −15° C. and this temperature was maintained during treatment. Samples were thawed by standing in water at 37° C. Suitable dilutions were made from all samples and used for surface plate counts.

Counting technique

Because the food media were irradiated before inoculation to remove contaminants, the requirement was merely to count salmonellae in the absence of other organisms. Suitable dilutions were made with quarter-strength Ringer's solution. The surface-plate count technique used throughout was a modified form of the method of Miles & Misra (1938) with 'Oxoid' nutrient agar as the medium. Initial runs were carried out to reveal which medium gave the best recovery for salmonellae after irradiation. Apart from 'Oxoid' nutrient agar, the media tested were deoxycholate-citrate agar, MacConkey agar and 8% horse blood agar. The poorest recovery was on the selective medium deoxycholate-citrate agar; there was no significant difference in numbers recovered on the other three media. 'Oxoid' nutrient agar did, however, produce the most reproducible results and the largest colonies, and was the simplest to prepare; it was therefore used for the dose/survival curve experiments.

Construction of survival curves

Three dose/survival curves were obtained separately for each of the systems examined. A common regression line was fitted to these by means of the method of least squares with an electronic computer. The regression line is of the form $y = ax + b$, where y is the logarithm of the surviving fraction, x is the dose of radiation, a is the slope of the line and b is the logarithm of the extrapolation number (Alper, Gillies & Elkind, 1960).

Radiation source

Gamma radiation from a 1000-curie Cobalt-60 source was used throughout. The source is a modified form of the 'hot spot' described by Eastwood, 1955; the dose rate was 0·25 Mrad./hr.

Large-scale experiments—artificially and naturally contaminated products

Direct evidence of the effectiveness of radiation in eliminating salmonellae from frozen horse-meat, desiccated coconut and bone meal was obtained by treating samples known to be naturally contaminated with salmonellae; this was followed by bacteriological examination. Frozen whole egg was heavily contaminated by artificial inoculation before irradiation.

(*a*) *Whole egg*

(i) During experiments described by Heller *et al.* 1962, on the pasteurization of whole egg, 1000 gal. of liquid egg held in the mixing tank at a commercial plant were inoculated with *S. gallinarum* to give a final salmonella count of approximately 500,000/ml. Samples were withdrawn, canned and frozen (−15° C.) at the plant, and used for radiation experiments. The cans were maintained frozen during both transport and irradiation with a dose of 0·5 Mrad., which was applied within 48 hr. of the inoculation. General colony, coliform and salmonella counts were made at all stages of the experiments.

In a similar experiment carried out earlier, *S. typhi-murium* phage-type 2b was used; again the final salmonella count was approximately 500,000/ml., but the dose used was only 0·35 Mrad.

(ii) In an earlier experiment (Hobbs, Horne & Ingram, unpublished) carried out at a different plant, bulked liquid egg was inoculated with a mixture of *S. typhi-murium*, *S. thompson* and *S. senftenberg*, to give counts of approximately 500/ml. of each. This concentration was chosen to represent the numbers which might be encountered in practice in a badly contaminated commercial sample. The infected egg was filled into 28 lb. cans, frozen (−15° C.) and irradiated at 0·5 Mrad. From some of the cans 60 g. frozen samples were removed by the method described by Hobbs & Smith (1955), and examined for salmonellae. The tests were carried out over a period of 3 months, during which time the frozen egg was maintained at −10° C.

(*b*) *Frozen horse-meat*

Thirty-five tons of frozen horse-meat from a consignment at the Port of London, known to be contaminated with salmonellae, was transported by road in an unrefrigerated vehicle (3 hr.) to Wantage Research Laboratory, in a series of batches of 5 tons each. The material was still frozen hard on arrival. At the Port the large blocks had been sawn up into smaller blocks, each approximately 1 cu.ft., to fit the conveyor system of the Cobalt-60 Package Irradiation Plant. In order to avoid thawing of the meat and the possibility of drip during passage through the plant, the dose used was 0·25 Mrad., so that the exposure time in the irradiation

chamber was no more than a few hours. After treatment the meat, still frozen, was returned to a refrigerated store at the Port.

A large number of 200 g. samples were taken at random from each batch both before and after irradiation. The samples were sawn from different blocks, instruments being sterilized after each sample. The samples were packed in polythene bags, heat-sealed, and maintained frozen until examined for salmonellae.

(*c*) *Desiccated coconut*

A series of 50 g. samples of coconut was taken from a 100 lb. bag found to be positive for salmonellae on arrival at the Port of London. The samples were canned, divided into batches, and irradiated at a series of doses up to 0·55 Mrad. As well as examination for salmonellae, coliform and general colony counts were performed.

In a later experiment another contaminated bag was sampled similarly, and a number of samples were irradiated at one dose level of 0·45 Mrad. Counts and examination for salmonellae were performed in the same way.

(*d*) *Bone meal*

A series of 10 g. samples were taken from a sack of bone meal known to be contaminated with salmonellae, and double-packed in plastic bags. Nine samples were treated at each of a series of doses up to 0·75 Mrad. Coliform and general colony counts were carried out as well as an examination for salmonellae.

Bacteriological examination

Plate and coliform counts were carried out on most samples before and after irradiation. The surface drop technique on horse blood agar incubated at 37° C. for 24–48 hr. was used for colony counts from suitable dilutions of a suspension of 10 g. of the food in 100 ml. quarter-strength Ringer's solution. Duplicate 1/10 and 1/100 dilutions were inoculated into single strength MacConkey broth for coliform organisms at 37° C. Tubes showing acid and gas were subcultured into brilliant green bile salt broth (Mackenzie, Taylor & Gilbert, 1948), and peptone water, both at 44° C., for gas and indole production by *Escherichia coli*.

The surface drop technique on suitable media was used also for salmonella counts, and in addition a dilution method similar to that used for estimating the probable number of coliform bacilli in water (Report, 1961*a*), Selenite F or nutrient broth being substituted for MacConkey broth. In most instances at least 50 g. of each sample were examined for the presence of salmonellae. In addition, 25 g. quantities were inoculated into each of two of the liquid enrichment media, Selenite F, tetrathionate and nutrient broth. After 24 hr. and 72 hr. at 37° C. each enrichment culture was subcultured on to the two selective agar media, deoxycholate-citrate and Wilson and Blair's medium, and incubated at 37° C. for 2 days. Characteristic colonies were picked for fermentation reactions into the Gillies (1956) modification of Kohn's (1954) tubes and also on to MacConkey agar for purity and lactose fermentation. Slide agglutination was used for serological identification; obscure serotypes were sent to the Salmonella Reference Laboratory, and where

phage typing was applicable the strains were sent to the Enteric Reference Laboratory.

It was recognized that more serotypes of salmonellae may have been present in the samples, but the extra time involved in picking and identification of more colonies or in the application of a technique such as that described by Harvey & Price (1961, 1962) was considered not to be justifiable.

Treatment by irradiation may have affected surviving organisms in some way so that different methods of identification would be required, but so far ther is no direct evidence to suggest that irradiation damage to micro-organisms makes different isolation techniques necessary.

Radiation source

The Spent Fuel Rod Assembly at A.E.R.E., Harwell, was used throughout as source of gamma radiation, except for the treatment of frozen horse-meat; the dose rate was approximately 1·5 Mrad/hr. The horse-meat was treated in the Cobalt-60 Package Irradiation Plant at Wantage, which has been described in detail by Jefferson, Rogers & Murray (1961).

RESULTS

The dose/survival curves obtained with the various media, illustrated for *S. typhi-murium* in Fig. 1, were treated as being strictly exponential, and the *D* value (dose required to reduce the number of survivors to one-tenth) for each system was calculated directly. Few of the curves extrapolated back to the ordinate to 100 % survival, and therefore the dose required to reduce the population from 100 to 10 % is apparently lower than the *D* value which is given in Table 1 for each of the systems examined. However, the *D* value, which is applicable in all cases below a survival level of 30 %, is very useful for practical purposes; the dose needed to produce a given inactivation of salmonellae is calculated by multiplying the *D* value by the exponent of the required inactivation factor. For example, if an initial contamination of 100 orgs./g. is required to be reduced to only 1 org./1000 g., then the inactivation factor is 10^5 and the dose needed is $5 \times D$ value for the particular system. This dose would tend to be a little higher (about 8 % for *S. typhi-murium* in frozen horsemeat) than that needed if adjustment is made for the initial portion of the survival curve.

The serotypes varied in their radiation resistance; for example, *S. typhi-murium* was very significantly ($P < 0{\cdot}01$) more resistant than *S. senftenberg* in almost all the media. In fact it appeared to be the most radiation-resistant of the five serotypes examined, although *S. paratyphi* B was just significantly more resistant in frozen buffer and just significantly less in frozen horse-meat ($P = 0{\cdot}05$–$0{\cdot}02$). Salmonellae proved to be considerably more resistant in coconut or frozen horsemeat than in bone meal or frozen egg, and therefore no predictions regarding radiation resistance can be based on the general physical state of the food.

Though freezing had no effect on the resistance of the salmonellae in whole egg,

with the possible exception of *S. gallinarum*, it had a striking effect on the buffer suspensions, giving D.M.F.S (dose modifying factor = ratio of *D* value in the frozen state to *D* value unfrozen) of 1·6–2·8.

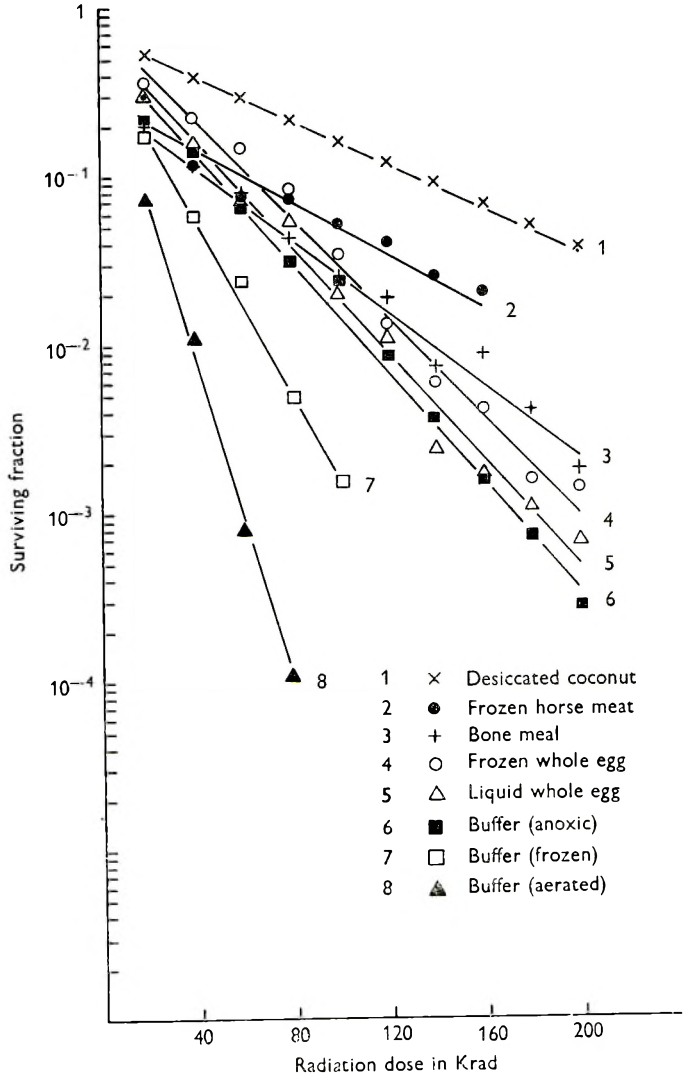

Fig. 1. Dose/survival curves for *S. typhi-murium* in various media.

The salmonellae were most radiation-sensitive in buffer suspension. There was no significant difference between aerated and non-aerated suspensions, but under anoxic conditions the D.M.F. was approximately 3.

The results in Table 2 illustrate the effectiveness of a dose of 0·5 Mrad. in eliminating a high degree of contamination with *S. gallinarum* in frozen egg. In fact, based on the *D* value for this serotype, only one survivor is to be expected in 2 l. of the egg—well beyond the practical limits of bacteriological detection. In an earlier experiment, the results after the inoculation of *S. typhi-murium* at the

same concentration gave positive results in 50 ml. samples, since this serotype is very much more resistant than *S. gallinarum* and the dose used was only 0·35 Mrad. A 10^5 inactivation is to be expected as calculated from the *D* value, and

Table 1. *The* D *values for various serotypes of salmonella in different media*

Media	*D* value (Krad.)* *S. gallinarum*	*S. senftenberg*	*S. typhi-murium*	*S. paratyphi* B	*S. meleagridis*
Liquid whole egg	43·0 (39·2–47·5)	50·4 (46·6–54·8)	63·2 (58·4–68·8)	—	—
Frozen whole egg	56·9 (49·1–67·8)	46·8 (44·1–49·8)	67·9 (63·4–72·9)	—	—
Frozen horse-meat	—	—	128 (112–148)	107 (97·2–120)	93·1 (83·5–105)
Desiccated coconut	—	134 (124–145)	158 (141–177)	—	—
Bone meal	—	55·7 (52·9–58·9)	91·0 (84·0–99·4)	—	—
Phosphate buffer					
Aerated	13·2 (12·3–14·3)	13·0 (12·7–13·3)	20·8 (19·1–22·9)	19·0 (17·0–21·5)	—
Non-aerated	13·2 (11·3–15·7)	12·9 (11·6–14·4)	17·7 (15·5–20·5)	17·1 (16·4–17·8)	—
Anoxic	36·3 (34·6–38·1)	38·9 (35·5–42·8)	61·9 (58·9–65·1)	65·9 (62·6–69·5)	—
Frozen	21·1 (19·2–23·3)	29·9 (28·1–32·0)	39·1 (35·2–43·8)	49·4 (40·4–63·6)	—

95 % confidence limits in brackets.
* Radiation dose required to reduce number of survivors to one-tenth

Table 2. *Bacterial examination of whole egg inoculated with* Salmonella gallinarum *and irradiated in the frozen state with* 0·5 *Mrad.*

Sample	General count/ml. at 37° C.	Coliform bacilli 0·1 ml.	Coliform bacilli 0·01 ml.	*S. gallinarum* count/ml.
Uninoculated, liquid	55,000	*	Faecal	Nil**
Inoculated, liquid	1,000,000	*	Faecal	400,000
Inoculated, frozen (i)	680,000	Faecal	Non-faecal	380,000
Inoculated, frozen (ii)	830,000	Faecal	Faecal	530,000
Inoculated, frozen irradiated (i)	< 500	Not found	Not found	Not found in 50 ml.
Inoculated, frozen irradiated (ii)	< 500	Not found	Not found	Not found in 50 ml.

* Not tested. ** *S. pullorum* present (< 5000/ml.)

this would result in five survivors/ml. This particular experiment with *S. typhi-murium* was carried out before *D* value figures were available. When the degree of contamination was only 500 orgs./ml. of each of three serotypes no salmonellae were detected in numerous samples tested after a dose of 0·5 Mrad., as the results

show in Table 3. *S. gallinarum* and *S. saint-paul*, which were not inoculated into the egg, were identified in the control samples, where they occurred as natural contaminants.

Table 3. *Salmonellae in whole egg inoculated with* Salmonella typhi-murium, S. thompson *and* S. senftenberg (500 *orgs./ml. of each*) *frozen and irradiated with* 0·5 *Mrad.*

Frozer egg sample	No. of cans examined	No. of cans in which were identified				
		S. typhi-murium	*S. thompson*	*S. senftenberg*	*S. gallinarum*	*S. saint-paul*
Uninoculated	116	21	0	0	42	1
Inoculated	30	30	30	30	*	*
Inoculated irradiated	40	0	0	0	0	0

* Not sought.

Table 4. *Irradiation of naturally contaminated frozen horse-meat*

Radiation dose (Mrad.)	No. of 200 g. samples examined	No. in which salmonellae detected
Nil–control	72	42*
0·25	57	Nil
0·75	15	Nil

* *S. oranienburg*, *S. derby*, *S. minnesota*, *S. meleagridis*, *S. poona*, *S. anatum*, *S. saint-paul*, *S. newport* identified.

Table 5. *Irradiation of naturally contaminated desiccated coconut*

	Radiation dose (Mrad.)	No. of 50 g. samples examined	No. in which Salmonellae detected	General colony count per g. at 37° C.	Coliform bacilli
Expt. I	Nil–control	18	18*	7000	Present in 0·1 g.
	0·1	9	8*	< 500	Not found in 0·1 g.
	0·2	9	6*	< 500	
	0·35	18	4*	< 500	
	0·45	9	Nil	—	—
	0·55	9	Nil	—	—
Expt. II	Nil–control	3	3**	< 500	Not found in 0·1 g.
	0·45	12	Nil	< 500	

* *S. bareilly* identified. ** *S. paratyphi* B identified.

A high natural contamination rate, about 60 %, was evident in the frozen horse-meat examined (Table 4), but a dose of 0·25 Mrad. was sufficient to give negative results in all the samples examined. *S. meleagridis* was identified in the controls; this has a high *D* value and only a 10^2 to 10^3 inactivation would be expected. This indicates that the number of contaminants was small.

All the unirradiated samples of desiccated coconut examined were positive for salmonellae (Table 5), and a dose of 0·45 Mrad. was required to give negative results by normal procedures. With bone meal (Table 6) the dose was rather higher—0·75 Mrad.—which was not to be expected, comparing the influence of coconut and bone meal on the radiation resistance of the same serotypes (Table 1). However, quite a wide range of serotypes were identified in the bone meal, not all of which have been examined for resistance. Another explanation is that the numbers of salmonellae initially present might have been very high; the initial general count was high and many organisms survived at 0·75 Mrad. The initial general counts with both the coconut and whole egg (uninoculated) were comparatively low, and with the former a dose of 0·1 Mrad. reduced the numbers to < 500/g.

Table 6. *Irradiation of naturally contaminated bone meal*

Radiation dose (Mrad.)	No. of 10 g. samples examined	No. in which Salmonellae detected	General colony count per g. at 37° C.	Coliform bacilli
Nil–control	9	6*	13 million	Present in 0·01 g. (faecal)
0·1	9	6**	675,000	Present in 0·1 g. (non-faecal)
0·25	9	6***	130,000	Present in 0·1 g. (non-faecal)
0·5	9	1****	7,500	Not found in 0·1 g.
0·75	9	Nil	2,750	Not found in 0·1 g.

* *S. oranienburg, S. cerro, S. meleagridis, S. kentucky, S. typhi-murium* phage-type 2d.
** *S. kentucky, S. chailey, S. minnesota.*
*** *S. california, S. oranienburg, S. bredeny, S. typhi-murium.*
**** *S. typhi-murium* phage-type 1b.

DISCUSSION

It is well known that various conditions of environment, such as temperature, media and availability of oxygen, influence the radiation sensitivity of bacteria, and this is again apparent from the results for salmonellae. The chemical composition of the media is very important; each of the foods exerts some protective effect compared with buffer and the effect can be very much greater than that produced even in an anoxic buffer suspension. No doubt a combination of protective effects contributes to the high dose requirement in certain media. It is necessary, therefore, in defining a suitable radiation process for the elimination of salmonellae, to consider each product concerned individually. This point was made by Erdman, Thatcher & MacQueen (1961) when they observed increased resistance in salmonellae and other micro-organisms of public health significance irradiated in broth compared with buffer.

The radiation dose to be recommended for the adequate elimination of salmonellae from the different products will depend on what degree of inactivation is considered safe on health grounds. This is extremely difficult to define; even a small number of salmonellae widespread throughout a commodity might lead to contamination of second vehicles such as cream cakes, trifles or cooked meat products, which are completed foods not requiring further heat treatment before consumption.

There is little information on the numbers of salmonellae in frozen or dried foods such as meat, egg products and coconut. However, the dilution technique performed from time to time has revealed figures of approximately 0·12–18/ml. for frozen whole egg, and < 0·03–0·072/g. for coconut; the highest count recorded for steamed bone flour was 120/g. (Report, 1959), but counts for meat and bone meal were usually < 0·3/g.; recorded figures for various animal feeds and ingredients were 0·02–0·08/g. (Report, 1961*b*). McCullough & Eisele (1951) in volunteer experiments gave 130,000 to 150,000 organisms as the lowest dose of salmonellae which could initiate illness. It is evident, therefore, that most of the incidents of salmonella food poisoning arising from the products considered in this paper are due to cross-contamination of other foods in which the salmonellae multiply, although cases of paratyphoid fever have been suspected to arise from the direct consumption of desiccated coconut (Anderson, 1960; Semple, Parry & Graham, 1961). The method of cross-contamination has been described for egg products (reviewed by Hobbs, 1963) and coconut (Wilson & Mackenzie, 1955), and must also occur from raw meat or poultry to cooked meat (Anderson, Galbraith & Taylor, 1961; Galbraith, Mawson, Maton & Stone 1962).

There is no doubt that a process which will considerably reduce the original number of salmonella contaminants will effectively contribute to the lessening of health hazards from this source. At a recent International Atomic Energy Agency (Vienna) panel meeting on the control of salmonellae using radiation (Report, 1963) it was recommended that the estimation of the effectiveness of radiation treatment should be based on the absence of salmonellae in, for example, 100 g. of product. This can easily be attained by a comparatively low dose of radiation when the numbers of initial contaminants are small; however, in order to deal with the possibility of large numbers being occasionally present, the dose should be as high as will allow economic operation of the process and yet avoid damage to the quality of the products.

Particular attention has previously been given to the use of radiation for the elimination of salmonellae from egg products, and a difference was noted in the radiation resistance of different serotypes. In studies on liquid whole egg (Proctor, Joslyn, Nickerson & Lockhart, 1953; Mossel, 1960) and on liquid and frozen egg white (Nickerson *et al.* 1957), *S. typhi-murium* was found to be more resistant than *S. senftenberg*, and the results presented in this paper show this to be so in a variety of media; in fact, *S. typhi-murium* was the most consistently resistant serotype examined. Brogle *et al.* (1957) found no such difference in resistance for the two serotypes referred to in whole egg solids, egg yolk solids or frozen egg yolk. A recent paper by Comer, Anderson & Garrard (1963) provides information on the radiation resistance of 18 serotypes of salmonella in frozen whole egg. Several of these were shown to be more resistant than *S. typhi-murium*, *S. give* being the most resistant. The culture of *S. typhi-murium* used was, however, less resistant than our own. For the purposes of this paper an estimate of the practical dose requirement for the treatment of the products currently reported is based on the resistance of *S. typhi-murium* phage type 2*c* (14).

Considering data for *S. typhi-murium* in frozen whole egg, a suitable practical

dose would be 0·5 Mrad., which gives a 10^7 inactivation as shown by D value determination, and should ensure the destruction of a variety of serotypes of salmonellae after inoculation into egg. This dose is somewhat higher than that quoted by other workers previously referred to for a similar egg product, and only 0·04 Mrad. less than that recommended by Comer *et al.* (1963). Treatment of frozen egg at 0·5 Mrad. has a negligible effect on the egg quality (Ley, Glew & Cornford, 1962), and the cost of the process is low (Ley & Rogers, 1962). Recently, however, a successful heat pasteurization process has been developed (Heller *et al.* 1962), and legislation is now nearing completion for the compulsory pasteurization of bulked whole egg. Heat and radiation processing of whole egg have been compared by Ingram, Rhodes & Ley (1961); radiation has an obvious advantage for the treatment of imported canned frozen egg as opposed to home-produced liquid egg, which can be heat-treated as such at 'breaking-out' stations before canning and freezing. Based on D value data only, the dose for egg in the liquid state would also be 0·5 Mrad. but this dose would be expected to have a detrimental effect on quality, such effects being observed at much lower doses by Mossel (1960).

At 0·64 Mrad. there is a 10^5 inactivation for frozen horse-meat, and this appears to be adequate in view of the absence of salmonellae from the naturally contaminated meat tested after treatment with as low a dose as 0·25 Mrad., when a 10^2 inactivation was obtained, although no *S. typhi-murium* were identified in the particular batches examined before irradiation. The application of 0·64 Mrad. for the treatment of horse-meat intended as pet food in the U.K. would be an economical and practical proposition (Ley, 1962). Experience in the operation of such a process would show whether the dose could safely be reduced below this level.

The microbiological data for desiccated coconut showed that 0·45 Mrad. reduced the number of salmonellae to a level which could not be detected; treatment with this dose gives an inactivation factor of 10^3. However, taste panel studies revealed that, even at this low dose level, change in flavour was detected as well as a slight darkening in colour, and the product was unacceptable to the trade (Glew & Ley, unpublished). Improved conditions of hygiene have considerably reduced the numbers of salmonellae in coconut and therefore the need for a large-scale process is not so pressing as hitherto.

For bone meal the effective dose was between 0·5 and 0·75 Mrad. Results indicate that natural contamination in this product is often heavy, and the practical dose will therefore be higher than for the other products investigated. A dose of 0·75 Mrad. is unlikely to affect the bone meal in any way, but the practicability of a radiation process for such a product remains to be determined. Animal feedingstuffs such as meat and fish meals might also be treated by radiation; it is quite feasible for a radiation plant to be designed to treat a variety of bagged feeds and fertilizers.

Though radiation is effective against salmonellae, the doses recommended for the treatment of individual products will also have a lethal effect on the other micro-organisms present, as revealed by the fall in the general count after treatment. The clostridia, however, are particularly radiation-resistant; it should therefore

be stressed that a radiation process aimed at salmonella elimination does not allow a change in the normal methods of storing and distributing the foods, e.g. at room temperature instead of frozen. The radiation resistance of various strains of staphylococcus in broth media, as shown by Erdman *et al.* (1961), is equal to or less than the resistance of the serotypes of salmonella which we have examined in foods; the numbers of staphylococci would therefore be very considerably reduced. Staphylococcal toxin is also affected; emetic activity for cats was reduced with a dose just greater than 0·1 Mrad. The same authors noted the high radiation sensitivity of coliforms, and our results, well illustrated with desiccated coconut, show that they are absent after low doses of radiation although salmonellae survived; this confirms that coliform destruction cannot be used as an indication of effective radiation treatment, although it is sometimes used as a measure of effective heat pasteurization.

Before any radiation process can be applied, evidence for the safety of irradiated food for consumption is required. A vast quantity of such evidence is now available (Report, 1962*b*), and it is not expected that the problem of wholesomeness will limit the use of the radiation process discussed in this paper.

SUMMARY

Studies on the use of gamma radiation for the elimination of salmonellae from whole egg, frozen horse-meat, desiccated coconut and bone meal show the extent to which the nature of the medium influences the resistance of these organisms to gamma radiation. There is also a variation in radiation resistance between different serotypes; *S. typhi-murium* was consistently the most resistant of those examined.

Based on experiments with artificially inoculated or naturally contaminated products, and also on dose/survival curve data, the dose requirement for the elimination of salmonellae from frozen whole egg is estimated at 0·5 Mrad., which gives a 10^7 reduction in numbers of *S. typhi-murium*; for frozen horsemeat 0·65 Mrad., giving a 10^5 reduction; and for bone meal between 0·5 and 0·75 Mrad., giving between 10^5 and 10^8 reduction. A dose of 0·45 Mrad. appears effective for desiccated coconut, with a reduction of 10^3, but such a radiation dose affects the quality of this product.

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