Vol. 65, No. 2 **June 1967**

THE JOURNAL OF HYGIENE

(Founded in 1901 by G. H. F. Nuttall, F.R.S.)

EDITED BY

R. M. FRY, M.A., M.R.C.S.

IN CONJUNCTION WITH

R. D. BARRY, M.A., B.V.Sc., Ph.D.

W. I. B. BEVERIDGE, D.V.Sc., M.A.

A. W. DOWNIE, M.D., F.R.S.

R. I. N. GREAVES, M.D., M.R.C.P.

J. O. IRWIN, M.A., D.Sc., Sc.D., F.S.S.

E. T. C. SPOONER, M.D., F.R.C.P.

Sir GRAHAM WILSON, M.D., LL.D., F.R.C.P., D.P.H.

CAMBRIDGE UNIVERSITY PRESS

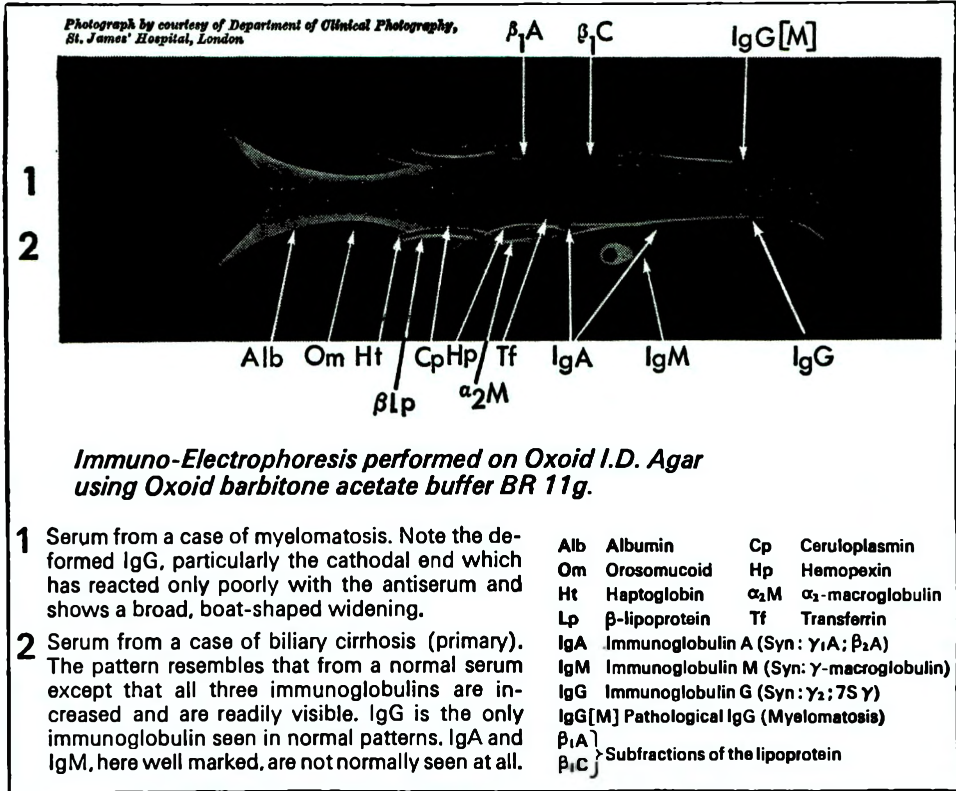
Bentley House, 200 Euston Road, London, N.W.1

American Branch: 32 East 57th Street, New York, N.Y.10022

Subscription per volume £4, 10*s*. ($17.50) (4 parts)

Single parts 32*s*. 6*d*. ($5.00)

A NEW PURIFIED AGAR FOR IMMUNO-ELECTROPHORESIS



Immuno-Electrophoresis performed on Oxoid I.D. Agar using Oxoid barbitone acetate buffer BR 11g.

1 Serum from a case of myelomatosis. Note the deformed IgG, particularly the cathodal end which has reacted only poorly with the antiserum and shows a broad, boat-shaped widening.

2 Serum from a case of biliary cirrhosis (primary). The pattern resembles that from a normal serum except that all three immunoglobulins are increased and are readily visible. IgG is the only immunoglobulin seen in normal patterns. IgA and IgM, here well marked, are not normally seen at all.

Alb	Albumin	**Cp**	Ceruloplasmin
Om	Orosomucoid	**Hp**	Hemopexin
Ht	Haptoglobin	**α_2M**	α_2-macroglobulin
Lp	β-lipoprotein	**Tf**	Transferrin

IgA Immunoglobulin A (Syn: γ_1A; β_2A)
IgM Immunoglobulin M (Syn: γ-macroglobulin)
IgG Immunoglobulin G (Syn: γ_2; 7S γ)
IgG[M] Pathological IgG (Myelomatosis)
β_1A, β_1C Subfractions of the lipoprotein

Oxoid I.D. Agar has been developed especially to meet the exacting requirements of Immuno-Electrophoresis. This technique demands an Agar of low conductivity, virtually free from chloride and sulphate ions. All pre-treatment, washing, etc., is eliminated.

CONVENIENT TABLET FORM Oxoid I.D. Agar comes in convenient tablet form and each tablet makes 50 ml. of Agar gel.

For a leaflet on the technique of Immuno-Electrophoresis on Agar and a comprehensive manual containing details of the Oxoid range, write to:
OXOID LIMITED, SOUTHWARK BRIDGE ROAD, LONDON, S.E.1.

for progressive laboratories

Recently Published

INSECTS AND HYGIENE

THE BIOLOGY AND CONTROL OF INSECT PESTS OF MEDICAL AND DOMESTIC IMPORTANCE

J. R. Busvine, Ph.D., F.R.I.Biol., Professor of Entomology as applied to Hygiene at the London School of Hygiene and Tropical Medicine.

This second edition has been extensively revised and rewritten, and is intended both for instructional and reference use by technologists concerned with insect pest control in temperate regions. The first part of the book summarizes different aspects of the subject, while the later chapters deal with 260 specific pests.

'Written with a nice appraisal of the dangers involved in chemical control methods, from straight toxic effects to the spread of resistant strains of insects. The "Practitioners" who use this guide should take due note of Professor Busvine's carefully weighed advice.' *British Book News*

£5

METHUEN **11 New Fetter Lane, London, E.C. 4**

Journal of General Virology

Editors: C. KAPLAN, Lister Institute of Preventive Medicine
& P. WILDY, The Medical School, Birmingham

Published for the Society of General Microbiology

A new journal in which papers are published on bacterial, plant and animal viruses, including studies of structure, genetics, systematics, pathogenesis and interactions with host cells. The aim of the journal is the rapid publication of research into the fundamental aspects of the study of viruses.

The first issue was published in January: a specimen copy will be supplied on request.

Subscription: £5. 10*s*. (U.S.A. $19.50) per volume of four parts. One volume in 1967.
Single Parts: 37*s*. 6*d*. (U.S.A. $6.50) plus postage.

CAMBRIDGE UNIVERSITY PRESS

Corrigendum

COLLIER & BLYTH, *J. Hyg., Camb.* (1966), **64**, 530.

In Table 2, under Experiment no. 6, the total particle count should read 10·2 in place of 11·9.

J. Hyg., Camb. (1967), **65**, 131
Printed in Great Britain

Estimating the date of infection from individual response times

BY G. G. MEYNELL
Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.1

AND TREVOR WILLIAMS
Department of Mathematics, Duke University, Durham, North Carolina, 27706, U.S.A.

(*Received* 3 *September* 1966)

It is characteristic of both naturally occurring and experimental infections that the affected individuals do not fall ill or die at the same time. If one defines the 'response time' of an individual as the interval between the earliest date on which he could have been exposed to infection (as by eating contaminated food) and the date on which he fell ill, then the distribution of individual response times is always skewed with a long tail to the right. The true distribution has often been taken as log-normal, since probit proportion of responses plotted against logarithm of time since exposure approximates to a straight line (Sartwell, 1950, 1952; Meynell & Meynell, 1958; Meynell, 1963). Sartwell (1966) pointed out that, if the true distribution is indeed log-normal, an unknown date of exposure, a_L, can be estimated from the dates of the individual responses by the method of quantiles (Aitchison & Brown, 1963, §6.24). This is so but, owing to the actual distributions observed in practice, the earliest date of exposure can be equally well estimated by another method, and it is shown here that the two estimates necessarily disagree.

Assuming first that the true distribution is log-normal, then it is clear from Fig. 1 that, because the plot of the distribution gives a straight line, the log individual response times corresponding to, say, 10% and 90% responses are symmetrically placed about the median response time for 50% responses. However, this will only be so when the earliest date of exposure is correctly chosen for, if this is taken as either before or after the real date, the corresponding plots in Fig. 1 are convex or concave upwards (see Aitchison & Brown, 1963, Fig. 6.3). In general, therefore, if b_2 is the calendar date of the median response time and b_1, b_3 are the dates corresponding to per cent responses, q and $100-q$, then the earliest date of exposure, a_L, is the solution of the equation

$$\log(b_2-a_L)-\log(b_1-a_L) = \log(b_3-a_L)-\log(b_2-a_L),$$

which is readily seen to be

$$a_L = \frac{b_1 b_3 - b_2^2}{b_1 + b_3 - 2b_2}. \quad (1)$$

The method can be illustrated by experiment 30 of Martin (1946) in which mice

ห้องสมุด กรมวิทยาศาสตร์

were injected intravenously with *Mycobacterium tuberculosis*. The numbers of mice found dead on successive days were 1, 0, 2, 6, 6, 6, 4, 5, 4, 0, 0, 1, 0, 0, 1; the first death occurred 15 days after challenge; and all the inoculated mice died. Suppose all that is known is the number of mice dying on a given day of the month. If the first mouse is assumed to have died on the 20th, a plot of proportion of mice dying against calendar date shows the median date (b_2) to be 24·6 and the dates (b_1, b_3) corresponding to 10% and 90% deaths to be 21·8 and 28·2. Hence from (1)

$$a_L = 12.$$

That is, the estimated date of infection was the 12th compared to the assumed date, the 5th.*

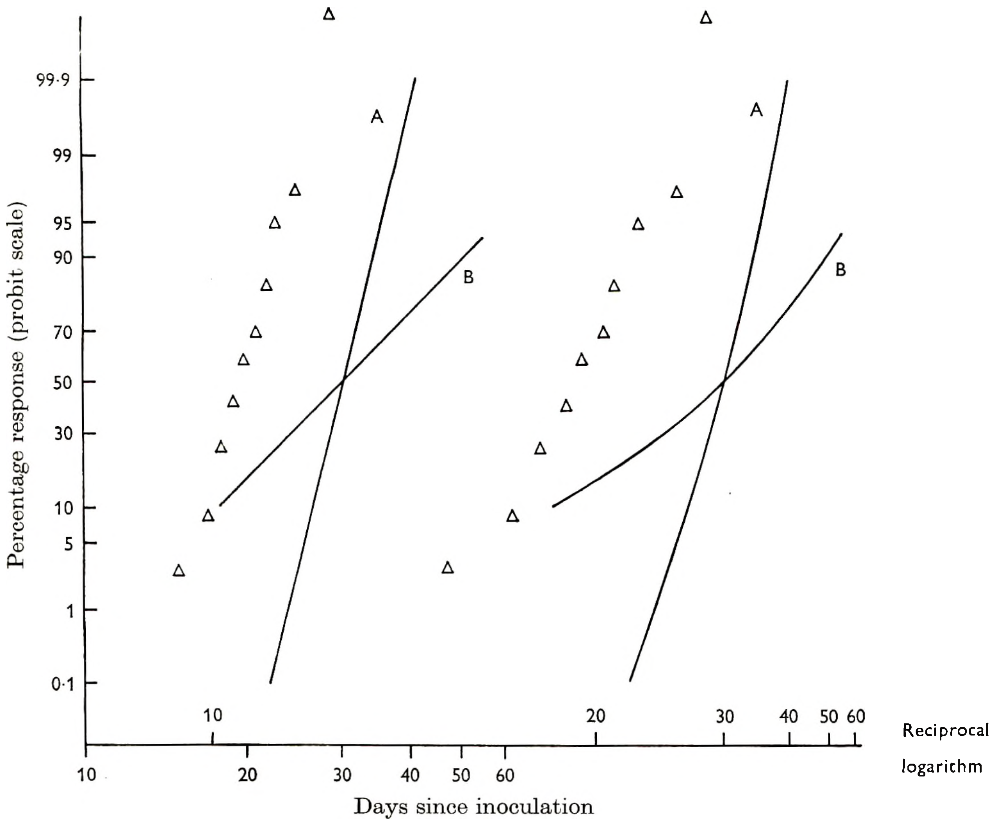


Fig. 1. Three distributions of response times plotted with either logarithm or reciprocal of time since inoculation (curves on left and right of figure, respectively). Ordinate: percentage responses, on probit scale. Abscissa: logarithmic time below, reciprocal time above. △, Experiment 20 of Martin (1946). Curves *A* and *B*, log-normal distributions with dispersion factors of 1·1 and 1·5 respectively.

* Even when the true distribution is log-normal, the estimation of a is recognized as being imprecise (Aitchison & Brown, 1963). In using quantiles, the values of b_1, b_2, and b_3 were obtained from a curve fitted to the observations. However, a can also be estimated directly from the data by Cohen's method (Aitchison & Brown, 1963, § 6.22). This was done here, but, although Martin's experiment was deliberately selected as an example because it appeared log-normal on probit paper, the estimate of a was wildly inaccurate, namely, more than 210 days before the first death.

It will be seen that the estimation of a_L depends, not upon the logarithm of the individual response time being normally distributed when measured from the correct time origin, but merely upon it being *symmetrically* distributed. The logarithm is far from being the only suitable function for, in many infections, the reciprocal also appears to be symmetrically distributed (Cavalli & Magni, 1943; Bryan, 1957). Host-pathogen systems are highly unlikely to fall into two fundamentally different classes, corresponding to the two transformations. It seems far more probable that many data can be fitted equally well in either way. On examination, this proved to be so, the reason being that, although response times are distributed, they always fall fairly close to the mean. Assuming a log-normal distribution, the degree of scatter can be expressed by the 'dispersion factor' (Sartwell, 1950) = time corresponding to 84% response/median response time. Its minimum value is 1·0, observed if all the hosts respond simultaneously, and the observed value rarely exceeds 1·5 in either epidemics (Sartwell, 1950, 1952, 1966; Williams, 1965) or experimental infections, and is often less. Given such small values, it is apparent from Fig. 1 that the corresponding log-normal distributions will appear virtually linear when plotted against reciprocal time, and, furthermore, that it will be almost impossible to determine which distribution truly describes the observations, remembering that these are always somewhat erratic and that the sample sizes tend to be small. Assuming the reciprocal of individual response time to be symmetrically distributed and the time of infection, a_R, correctly chosen, we have

$$\frac{1}{b_2-a_R}-\frac{1}{b_1-a_R}=\frac{1}{b_3-a_R}-\frac{1}{b_2-a_R}$$

whose solution is

$$a_R = \frac{2b_1b_3-b_2(b_1+b_3)}{b_1+b_3-2b_2}. \tag{2}$$

In Martin's experiment, $a_R = -0{\cdot}6$. That is, the date of infection is given as 0·6 days before the 1st of the month.

Neither estimate is accurate to a useful degree in this example, but, quite apart from this, the whole procedure is open to a fundamental objection. We have seen that either the logarithm or the reciprocal of time can be used to estimate a. However, comparison of equations (1) and (2) shows

$$a_R = 2a_L-b_2.$$

In other words, a_R and a_L must always differ. The objection to these procedures is, therefore, that although either method is valid in itself, neither is of practical value because there is at present no reason *a priori* to accept one rather than the other.

SUMMARY

In principle, an unknown date of infection can be estimated from individual response times, provided some function of these with suitable origin is symmetrically distributed. Observed times are always skewly distributed, and either logarithm or reciprocal of time can be used to produce symmetry. Either is equally

justifiable but the resulting estimates are not only very imprecise but are also inconsistent, so that neither is of practical value.

One of the authors (T.W.) wishes to acknowledge the support of grant no. GM AI 13491–01 of the U.S. National Institutes of Health.

REFERENCES

AITCHISON, J. & BROWN, J. A. C. (1963). *The Lognormal Distribution.* Cambridge University Press.

BRYAN, W. R. (1957). Interpretation of host response in quantitative studies on animal viruses. *Ann. N.Y. Acad. Sci.* **69**, 698.

CAVALLI, L. & MAGNI, G. (1943). Quantitative Untersuchungen über die Virulenz. III. Mitteilung: Analyse der Häufigkeitsverteilung der Absterbezeiten von infizierten Mäusen. *Zentbl. Bakt. ParasitKde, Abt.* 1, *Orig.* **150**, 353.

MARTIN, A. R. (1946). The use of mice in the examination of drugs for chemotherapeutic activity against *Mycobacterium tuberculosis. J. Path. Bact.* **58**, 580.

MEYNELL, G. G. (1963). Interpretation of distributions of individual response times in microbial infections. *Nature, Lond.* **198**, 970.

MEYNELL, G. G. & MEYNELL, E. W. (1958). The growth of micro-organisms *in vivo* with particular reference to the relation between dose and latent period. *J. Hyg., Camb.* **56**, 323.

SARTWELL, P. E. (1950). The distribution of incubation periods of infectious disease. *Am. J. Hyg.* **51**, 310.

SARTWELL, P. E. (1952). The incubation period of poliomyelitis. *Am. J. publ. Hlth* **42**, 1403.

SARTWELL, P. E. (1966). The incubation period and the dynamics of infectious disease. *Am. J. Epidem.* **83**, 204.

WILLIAMS, T. (1965). The basic birth-death model for microbial infections. *Jl R. statist. Soc.* **27**, 338.

J. Hyg., Camb. (1967), **65**, 135
With 1 *plate*
Printed in Great Britain

Attenuation of virulence in *Vibrio cholerae*

BY K. BHASKARAN AND V. B. SINHA
Department of Microbiology, Central Drug Research Institute, Lucknow, India

(*Received* 18 *November* 1966)

Results of recent field trials have exposed the limitations of present-day cholera vaccines for conferring immunity to man (Cvjetanovic, 1966). A summary of the results obtained in different field trials is given in Table 7. As these vaccines were made up of mixtures of killed cells of the two antigenic types, Inaba and Ogawa, of *Vibrio cholerae* or of *V. el Tor*, there is speculation whether live vaccines may prove superior (Panse & Dutta, 1964). There is also a revival of the concept, originally put forward by Besredka (1927), that local immunity in the intestinal tract might play an important part in immunity to cholera, which is predominantly an intestinal infection without systemic invasion (Burrows, Elliott & Havens, 1947; Freter, 1956, 1965; Mukerjee, 1963).

The development of any live vaccine, whether for oral or parenteral administration, should necessarily involve a detailed study of attenuation of virulence. The attenuated nature of certain strains of *V. el Tor*, isolated from the natural waters of the Middle East and India, has been described (Mukerjee, 1963). The present paper describes the isolation and study of an attenuated mutant of *V. cholerae*. The results and the current evidence which warrants trials of live cholera vaccine will be discussed.

MATERIALS AND METHODS

Characterization of strains

From *Vibrio cholerae*, strain 162/p, a purine-requiring attenuated mutant (strain A11) was initially isolated with the aid of N-methyl-N′-nitro-N-nitrosoguanidine (NTG). This mutant was further characterized with additional nutritional deficiencies and streptomycin resistance, to yield strain C14–S5, which was employed in this study along with the parent strain 162/p. The sequence of mutant isolations and the markers of each can be seen in Table 1. In other respects, all the strains were typical of *V. cholerae*, O Group I (Gardner & Venkatraman, 1935), possessing the following characters: gram negative curved motile rods, liquifying gelatin, fermenting mannose and saccharose but not arabinose (Group I, Heiberg, 1935), non-haemolytic to sheep cells, sensitive to Group IV phage (Mukerjee, 1961) and not agglutinating sheep red cells (Barua & Mukherjee, 1963). The colonies formed by the attenuated mutants on nutrient agar were much smaller than those of the parent strain, 162/p, and were therefore designated dwarf colony types (see Table 1).

For agglutination and agglutinin absorption tests, an Inaba type mutant of 162/p (strain 162/p-IN) was isolated by the technique of Shrivastava & White (1947), and a rough mutant (strain A69) by treatment with NTG.

All strains were maintained on nutrient agar slants in the refrigerator at 4° C. For the experiments, transfers were made on the same medium and incubated overnight at 37° C. This was used as seed for inoculating nutrient broth when required. All cultures were grown at 37° C.

Table 1. Vibrio cholerae, *strain 162/p, and its attenuated mutants*

Strain designation	Markers	Origin	Technique employed for isolation
162/p	Prototroph, Ogawa, virulent	162 (pur^-) (wild type)	Selection
A 11	pur^-, Ogawa, dwarf colony type*, attenuated	Mutant of 162/p	NTG
B 16	pur^-, nic^-, Ogawa, dwarf colony type*, attenuated	Mutant of A 11	NTG
C 14	pur^-, (ser^-), nic^-, Ogawa dwarf colony type*, attenuated	Mutant of B 16	NTG
C 14–S 5	pur^-, (ser^-), nic^-, str-r, Ogawa, dwarf colony type*, attenuated	Mutant of C 14	Selection

pur^-, Requirement for purine (satisfied by hypoxanthine); nic^-, requirement for nicotinic acid; (ser^-), requirement for serine (partial); str-r, resistance to streptomycin (500 μg/ml.); NTG, treatment with N-methyl-N′-nitro-N-nitrosoguanidine.

* Dwarf colonies (after 24 hr. incubation at 37° C.) had an average diameter of 1·57 mm. on nutrient agar and 2·63 mm. on brain heart infusion agar (Difco): colony size of the parent strain (162/p) were 3·45 mm., and 4·1 mm. respectively on these media.

Isolation of mutants by treatment with N-methyl-N′nitro-N-nitrosoguanidine (NTG)

Ten ml. of a 4 hr. nutrient culture of the strain were centrifuged and the bacterial deposit was suspended in 1 ml. of physiological saline (pH 6·0). To this (with a viable count of $3–5 \times 10^9$ organisms), 0·1 ml. of a 0·4 % freshly prepared aqueous solution of NTG (Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.) was added and incubated for 30 min. Later, 10 ml. of broth were added and the culture was centrifuged. The deposit was resuspended in 10 ml. of fresh nutrient broth and incubated for 4 hr. The culture thus obtained was seeded on nutrient agar plates, so as to give discrete colonies after 24 hr. incubation. Colonies thus isolated were tested for mutant characters.

Culture media

Nutrient broth contained, per litre of distilled water: peptone (Oxoid), 10 g.; yeast extract (Oxoid), 2·5 g.; and NaCl, 5 g. It was adjusted to pH 8·5 before sterilization. For nutrient agar, nutrient broth was solidified with 1 % agar powder (Oxoid, no. 3).

Minimal medium contained per litre of distilled water: K_2HPO_4, 7 g.; KH_2PO_4, 3 g.; NaCl, 5 g.; $Na_3C_6H_5O_7.2H_2O$, 0·5 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4.7H_2O$, 0·1 g.; and DL-methionine, 0·1 g. The pH of the medium was 7·2. Sterile 50 % glucose solution was added to give a final concentration of 0·1 % before use. The medium was solidified, when required, by the addition of 1 % Agar powder (Oxoid, no. 3).

Other experimental details are described in the text where required.

RESULTS

Agglutination and agglutinin absorption tests

As this study was intended to demonstrate attenuation of virulence in *V. cholerae*, while retaining full antigenicity, it was essential to prove that the observed differences between parent and mutant strains did not result from smooth to rough mutations in the organism. Rough mutants of *V. cholerae* are known to be avirulent to animals, and a comparative virulence study of rough and attenuated mutants (strains A 69 and A 11 respectively) of 162/p was described briefly elsewhere (Bhaskaran & Sinha, 1966).

The procedure employed was to carry out agglutination and agglutinin absorption tests to detect the presence of rough O antigens in the strains under study. O antisera were obtained from rabbits, immunized with heat-killed suspensions of 162/p, C 14–S 5 and A 69 (rough), and their agglutinin titres were determined before and after absorption with homologous and heterologous strains.

For preparing the antisera, adult male rabbits (1·5–2·0 kg.) received six 1 ml. intravenous doses of heat killed suspensions of overnight nutrient agar cultures (approximately 5×10^9 organisms) over a period of 11 days. The animals were bled on the 17th day and sera, after separation, were stored at 4° C. without preservative. For titration of agglutinins the sera were diluted in saline, and saline suspensions of live cultures were used as antigens. The cell-serum mixtures were kept at 37° C. for 4 hr. and overnight at 4° C. before reading the results. In these tests, sodium chloride concentration was maintained at 0·85 %, except in tests with the rough strain when the salt concentration was reduced to 0·5 % to prevent auto-agglutination. Absorption of agglutinins was done by treating 0·1 ml. of each serum with 2·4 ml. of a dense live suspension in 0·85 % saline of the absorbing strain at 37° C. for 4 hr. and overnight at 4° C.

The titres before and after absorption are given in Table 2. It will be seen that O antisera prepared with 162/p and C 14–S 5 did not agglutinate the rough mutant (A 69) not were their agglutinins removed by it. Likewise, O antisera to A 69 neither agglutinated nor were absorbed by 162/p or C 14–S 5. Owing to incomplete absorption, a low residual titre was seen even after absorption with homologous strains in some cases. This does not however, influence the fact that the rough antigen was not detectable in 162/p and C 14–S 5, which were therefore typical smooth strains. As absorption with 162/p-IN did not significantly lower the titre of sera prepared with 162/p and C 14–S 5, the latter strains belonged to the Ogawa type. This is consistent with the present knowledge of O antigenic structure of *V. cholerae* which possess a common O antigen, and minor type-specific O antigens corresponding to Ogawa and Inaba types (Gardner & Venkatraman, 1935).

Mouse virulence

Although mice do not display the typical pathological manifestations of cholera, they succumb to intraperitoneal infection with *V. cholerae*. The infective dose may be considerably reduced if the organisms are suspended in mucin (Griffitts, 1942). This technique thus permits quantitative measurements of virulence of different

strains, and high mouse virulence is generally preferred in strains of *V. cholerae* (and *V. el Tor*) selected for use as vaccines in man.

In spite of the artificial nature of this experimental model, which may not be relevant to the disease in man or immunity to it (Panse, Jhala & Dutta, 1964), it seemed worth while to carry out experiments with 162/p and C14–S5 and determine how virulent they were to mice. Swiss mice (of either sex), 14–16 g. in weight, reared in this Institute, were distributed in groups of ten, each group receiving 0·5 ml. of graded decimal dilutions of a 4 hr. nutrient broth culture of the strain under study. All dilutions were made in nutrient broth, except that intended for

Table 2. *O agglutinin titres in immunized rabbits and agglutinin absorption tests*

Serum no.	Absorbed with	O agglutinin titre (Ogawa) 162/p	(Ogawa) C14–S5	(Inaba) 162/p-IN	(Rough) A69
1		5,000*	10,000*	500*	0*
	(Ogawa) 162/p	125	250	0	—
	(Ogawa) C14–S5	250	250	0	—
	(Inaba) 162/p-IN	2,000	5,000	0	—
	(Rough) A69	2,000	5,000	200	—
2		10,000*	10,000*	500*	0*
	(Ogawa) 162/p	250	250	0	—
	(Ogawa) C14–S5	250	250	0	—
	(Inaba) 162/p-IN	2,000	10,000	0	—
	(Rough) A69	5,000	10,000	500	—
3		2,000*	5,000*	500*	0*
	(Ogawa) 162/p	50	125	0	—
	(Ogawa) C14–S5	125	250	0	—
	(Inaba) 162/p-IN	1,000	2,000	0	—
	(Rough) A69	1,000	5,000	500	—
4		2,000*	5,000*	200*	0*
	(Ogawa) 162/p	0	50	0	—
	(Ogawa) C14–S5	50	125	0	—
	(Inaba) 162/p-IN	1,000	2,000	0	—
	(Rough) A69	1,000	2,000	200	—
5		0*	0*	0*	500*
	(Ogawa) 162/p	—	—	—	500
	(Ogawa) C14–S5	—	—	—	500
	(Inaba) 162/p-IN	—	—	—	500
	(Rough) A69	—	—	—	0
6		0*	0*	0*	1,000*
	(Ogawa) 162/p	—	—	—	1,000
	(Ogawa) C14–S5	—	—	—	1,000
	(Inaba) 162/p-IN	—	—	—	1,000
	(Rough) A69	—	—	—	50

* Titre of unabsorbed serum; 0, titre less than 50.

Serum nos. 1 and 2 derived from rabbits immunized with heat-killed suspensions of 162/p; serum no. 3 and 4 derived from rabbits immunized with heat-killed suspensions of C14–S5; serum no. 5 and 6 derived from rabbits immunized with heat-killed suspensions of A69.

intraperitoneal inoculation of mice, for which an aqueous suspension of 5 % mucin (Granular mucin, Type 1701-W, Wilson Laboratories, Chicago 9, Illinois, U.S.A.), adjusted to pH 7·2 after sterilization, was used as diluent.

Table 3. *Mouse virulence of strain 162/p and its attenuated mutant C14–S5*

Dose (0·5 ml.)	162/p Test no. 1 (2·6 × 10^8/ml.)	162/p Test no. 2 (4·6 × 10^8/ml.)	C14–S5 Test no. 1 (5·4 × 10^8/ml.)	C14–S5 Test no. 2 (2·2 × 10^8/ml.)
10^{-1}	—	—	7/10	5/10
10^{-2}	—	—	1/10	1/10
10^{-3}	—	—	1/10	0/10
10^{-4}	—	—	—	—
10^{-5}	10/10	—	—	—
10^{-6}	9/10	10/10	—	—
10^{-7}	7/10	10/10	—	—
LD50*	Less than 13 organisms	Less than 23 organisms	10^7 organisms	1·8 × 10^7 organisms

Figures in parentheses indicate viable counts of cultures employed. Numerator represents no. of deaths (in 48 hr.) and denominator the no. of animals in the group.

* Determined by the method of Reed & Muench (1938).

Table 3 records the results of this study. The LD 50 of C14–S5 was indeed very high (10^7 organisms and 1·8 × 10^7 organisms in two experiments), while 162/p proved to be highly virulent, the LD50 being < 13 and < 23 organisms in two experiments. Although vibrio strains with extremely high mouse virulence (with an LD50 of 1·69 and 1·90 organisms) have been reported (Pesigan, 1965), there does not appear to be any reference to a smooth strain of *V. cholerae* having such low mouse virulence as observed with C14–S5. In earlier experiments (Bhaskaran & Sinha, 1966), the LD50 of 162/p was estimated at about 111 organisms, and this was probably due to the use of a batch of mucin of different manufacture.

Virulence studies in adult and infant rabbits

The rabbit intestine is known to be suitable for the study of enteropathogenicity of *V. cholerae*, and infant rabbits, 10 to 16 days old, are particularly susceptible to intra-intestinal infection with *V. cholerae* or their toxic products (Dutta & Habbu, 1955; Oza & Dutta, 1963). Oral infection (and intoxication) is also possible (Feeley, 1965; Finklestein, Norris & Dutta, 1964). Diarrhoea starts 18–24 hr. after intra-intestinal infection and the animal shows signs of dehydration. Death occurs in 36–48 hr. and autopsy reveals a characteristic picture, in which a massive distension of the caecum with fluid resembling the rice-water stool of cholera in man is a constant finding.

Rabbits more than a month old are not susceptible to an experimental infection of this kind. Diarrhoea is not produced and the animal shows no abnormality. In these animals, however, if the organism is introduced in closed ileal loops of about 4 in. in length, created by ligatures at the ends, and the animal examined 24 hr. after infection, the loop presents a distended appearance due to the accumu-

lation of 14–20 ml. of fluid which is sometimes blood-stained (De & Chatterji, 1953). Choleragenic toxins also produce a similar effect (De, Ghose & Chandra, 1962; Finkelstein, 1965*b*). As many as four ileal loops may be infected in an animal, under ether anaesthesia after preliminary sedation with 'Intraval' Sodium (May and Baker Ltd., Dagenham, England).

These two experimental models were employed with advantage for the study of enteropathogenicity of 162/p and C14–S5. Adult rabbits (1·0–1·5 kg.) were employed for the ileal loop studies. Ten-day-old infant rabbits (albino), weighing between 100 and 130 g., were used for intra-intestinal infection which was done under ether anaesthesia by direct introduction of the organisms into the lumen of the small intestines of the animal after making an abdominal opening which was subsequently sutured. In these experiments, the dose employed for infection was 1 ml. of a 4 hr. nutrient broth culture, with a viable count of $3\text{–}5 \times 10^8$ organisms.

Table 4. *Experimental infection of adult and infant* (10 *day old*) *rabbits with strain* 162/*p and its attenuated mutant C* 14–*S* 5

	Adult rabbits			Infant rabbits		
Strain	No. of animals	No. of ileal loops infected	No. showing positive reaction	No. of animals infected	No. of animals exhibiting diarrhoea	No. of animals with fluid distension of caecum
162/p	17	25	21	15	12	14
C14–S5	8	22	0	15	0	0

Table 4 reveals the differences in enteropathogenicity observed between 162/p and C14–S5. Strain 162/p uniformly gave rise to ileal loop distension in adult rabbits, with the exception of four loops in two different animals, and C14–S5 gave consistently negative results. In many experiments, both the strains were used for infecting different loops in the same animal, and the contrast was striking (see Plate 1).

Of the 15 infant rabbits infected with 162/p, diarrhoea was observed in 12 animals after 24–40 hr. and autopsy performed on all the animals at the end of this period revealed the characteristic distension of caecum in 14 of them. Only 1 showed no signs of disease.

Similar experiments were performed with C14–S5, after this strain had been passaged through two infant rabbits in series, as such passages are known to enhance the virulence of *V. cholerae* strains to these animals (Dutta & Habbu, 1955). In the absence of diarrhoea or caecal distension, the passaged strain was reisolated by seeding the intestinal contents on nutrient agar containing streptomycin (500 μg./ml.). However, none of the 15 infant rabbits infected with C14–S5 (passaged strain) showed any evidence of diarrhoea. All the animals were killed after 24–40 hr. and autopsy revealed a normal appearance of the gut.

Chick embryo virulence

Gardner, Lyles, Lankford & Hagens (1963) showed that the 13 day chick embryo was well suited for the study of virulence of *V. cholerae* and that only smooth strains were virulent. Rough and smooth-rough (SR) variants were avirulent to the chick embryo. If deaths were scored 24 hr. after allantoic inoculation, reproducible LD 50 titrations of virulence of different strains were possible. Compared to *V. cholerae*, strains of *V. el Tor* showed an extremely high virulence (Finkelstein, 1964). Factors determining the resistance of older chick embryos to allantoic infection with *V. cholerae* have also been elucidated (Finkelstein & Ramm, 1962).

It was therefore expected that the difference in virulence observed between 162/p and C 14–S 5 in mouse and rabbit would be further confirmed in the chick embryo. Twelve-day-old White Leghorn eggs were inoculated allantoically with 0·1 ml. of decimal dilutions in saline of 4 hr. broth cultures of the strains. The infected eggs were incubated at 37° C. and examined 24 hr. later for viability. Results are shown in Table 5.

Table 5. *Chick embryo virulence of strain* 162/*p and its attenuated mutant* *C* 14–*S* 5

Dose (0·1 ml.)	Experiment no. 1		Experiment No. 2		Experiment no. 3	
	162/p ($2·33 \times 10^8$/ ml.)	C 14–S 5 ($2·26 \times 10^8$/ ml.)	162/p (2×10^8/ ml.)	C 14–S 5 (3×10^8/ ml.)	162/p ($2·8 \times 10^8$/ ml.)	C 14–S 5 ($5·7 \times 10^8$/ ml.)
10^{-1}	n.d.	1/8	n.d.	1/8	n.d.	2/8
10^{-2}	7/8	1/8	6/8	0/8	8/8	1/8
10^{-3}	4/8	0/8	7/8	0/8	6/8	1/8
10^{-4}	5/8	n.d.	7/8	n.d.	6/8	n.d.
10^{-5}	1/8	n.d.	4/8	n.d.	5/8	n.d.

Figures in parentheses indicate viable counts of the cultures employed. Numerator represents deaths and denominator the total number of embryos infected. n.d., not done.

The difference in virulence of the two strains was confirmed. But a precise dose/effect relationship was not obvious, especially with strain 162/p, and this was possibly owing to the use of 12-day instead of 13-day embryos, or to a slight variation in the age of the embryos at the time of supply. As each of the three experiments recorded in Table 5 was done with a single lot of eggs received on the day of the experiment, and distributed at random into groups of 8, it is extremely unlikely that any shift to virulence with C 14–S 5 will be observed even with stricter control of the age of the embryos in this test.

Population changes of 162/*p and C* 14–*S* 5 in vivo

It would be logical to expect that the ability of 162/p to produce fluid distension of ileal loops of adult rabbits, cholera-like disease in infant rabbits, and death of mice and chick embryos, might be due to the active multiplication of the organism *in vivo*, perhaps associated with the release of toxic factors. In the same way, the uniform pattern of avirulence of C 14–S 5 in the four different models may be an

indication of the inability of this strain to multiply in the tissues of the animal to an extent required for causing the pathological changes, or of its non-toxicity. Its inability to multiply is suggested by the peculiar dwarf colony character of C14–S5 which reflected its slow rate of growth. Although precise growth studies have not yet been carried out, the growth of C14–S5 in fluid media like nutrient broth is much slower than 162/p especially from small inocula. If this is due to different optimal conditions for the growth of C14–S5, the specific requirements are yet obscure.

With a view to studying the *in vivo* growth of 162/p and C14–S5 in the experimental models investigated, viable counts were performed 24 hr. after infection and were compared with the initial numbers employed for setting up the infection. Such estimates were made in closed ileal loops of adult rabbits, and in the intestinal tract of 10-day-old infant rabbits (Table 6).

Table 6. *Growth and/or survival of strain* 162/*p and its attenuated mutant C*14–*S*5 *in the ileal loops of adult rabbits and in the intestinal tract of infant* (10-*day-old*) *rabbits*

Experiment	Strain	Viable count: Infecting dose	Viable count: 24 hr. after infection	Ratio final count / initial count
Ileal loop (adult rabbits)	162/p (test no. 2)	$5{\cdot}6 \times 10^8$	$8{\cdot}3 \times 10^9$	14·82
	162/p (test no. 2)	$4{\cdot}4 \times 10^8$	$1{\cdot}9 \times 10^{10}$	43·18
	C14–S5 (test no. 1)	$5{\cdot}4 \times 10^8$	$2{\cdot}0 \times 10^9$	3·70
	C14–S5 (test no. 2)	$4{\cdot}9 \times 10^8$	$7{\cdot}9 \times 10^7$	0·16
Intra-intestinal infection (infant rabbits)	162/p (test no. 1)	$2{\cdot}23 \times 10^8$	$6{\cdot}0 \times 10^{10}$	269·10
	162/p (test no. 2)	$2{\cdot}23 \times 10^8$	$3{\cdot}0 \times 10^{11}$	1345·00
	C14–S5 (test no. 1)	$2{\cdot}15 \times 10^8$	$7{\cdot}4 \times 10^7$	0·34
	C14–S5 (test no. 2)	$2{\cdot}15 \times 10^8$	$7{\cdot}0 \times 10^7$	0·33

As expected, experiments with 162/p gave rise to pathological reactions in both, whereas with C14–S5 they were absent. For the counts, each ileal loop was dissected out and disintegrated in a liquidiser (Kenwood Manf. Co. Ltd., Woking, England). The material thus obtained was suspended in 100 ml. of nutrient broth, and dilutions of this in broth were seeded on nutrient agar plates and the colonies counted after 24 hr. at 37° C. From the counts obtained, an approximate estimate of the total number of viable organisms of the test strain was made. In the case of infant rabbits, the entire gut (small and large intestines) was removed and disintegrated and total viable counts were performed as described above.

Strain 162/p so far outgrew the normal intestinal flora that these caused no difficulty in the colony counts, but with C14–S5, which did not multiply so freely,

counts had to be performed on nutrient agar containing streptomycin (500 μg./ml.) to inhibit the normal flora.

Table 6 shows the wide divergence between the two strains. The number of viable organisms of 162/p, 24 hr. after infection, was considerably in excess of the input, especially in the gut of infant rabbits. With C14–S5, the final counts were generally less, except in one animal, which registered a small rise. As non-viable cells were automatically excluded from these estimates, the final counts provided only an approximate estimate of the extent of bacterial growth. Even though C14–S5 did not register a significant rise in numbers during the 24 hr. period, the final counts varied from one-fifth to one-third of the input in three animals, which showed the absence of any rapid clearing mechanisms in the host. As infant rabbits, infected with C14–S5, did not pass any faecal matter during the 24 hr. period, this mode of elimination of the organism was excluded, but with 162/p there must have been loss through diarrhoea occurring in the infant rabbits. These considerations do not, of course, apply to the closed ileal loops in adult rabbits.

DISCUSSION

In this study, the term virulence, as applied to *V. cholerae*, is used in the general sense to mean the ability to cause either death by rapid proliferation or characteristic pathological manifestations in experimental animals, regardless of any toxic factors. In fact, a variety of toxic factors may be demonstrated in *V. cholerae* and differentiated from one another by their association with different parts of the cell, dialysability, and various toxicities demonstrable in mice, chick embryos, adult and infant rabbits, cell culture lines and anurian epithelium (Burrows, 1965). More is now known regarding the choleragenic toxin (choleragen), which has been identified as a protein and which is probably released during active proliferation of *V. cholerae* under optimal conditions. Of particular interest is the observation that neither endotoxin nor mucinase play any significant role in the pathogenesis of cholera. These advances are the result of some noteworthy research in this field by Finkelstein and his colleagues (Finkelstein *et al.* 1964; Finkelstein, 1965*a*, *b*; Finkelstein, Atthasampunna, Chulasamaya & Charunmethee, 1966; Finkelstein, Sobocinski, Atthasampunna & Charunmethee, 1966).

While Mukherjee (1963) identified apathogenic strains of *V. el Tor*, with a certain degree of residual virulence, in natural isolates from water sources, we have in this work used the chemical mutagen, N-methyl-N′-nitro-N-nitrosoguanidine, for the isolation of an attenuated mutant of *V. cholerae*, and subsequent labelling with marked characters. A variety of other mutants have also been isolated with this agent, as well as with ethyl methane sulphonate (Bhaskaran, Sinha & Iyer, 1966).

The attenuated mutant C14–S5 is a slow growing strain, giving rise to dwarf colonies on nutrient agar, a frailty that possibly accounts for its non-pathogenicity to mouse, rabbit and chick embryo. The capacity of this strain to elaborate the choleragenic toxin and other toxicities during growth remains to be studied. Antigenic analysis excluded the possibility of roughness in the strain. Further,

C14–S5 is as effective as wild type strains when employed as live vaccine in mouse protection tests (Bhaskaran & Sinha, unpublished observations) carried out as recommended by Pittman & Feeley (1965).

The stability of the attenuated mutant, C14–S5, and its predecessors (A11, B16 and C14) seems to be well established. These strains have been under study for over 6 months, during which a number of transfers have been carried out on nutrient media and a variety of animal experiments have been performed. At all times, the dwarf colony character and attenuation have remained a constant feature of these mutants. Whether the genetic determinants of virulence and normal growth characteristics can be transferred to the attenuated mutants by conjugation with wild type *V. cholerae* strains is not known. With the recognition of a fertility factor in *V. cholerae* (Bhaskaran, 1960, 1964) such studies should be possible.

Recent field trials have shown that killed cholera vaccines are of low efficacy in man, with the exception of a few which however caused severe reactions (see Table 7). In the laboratory there is convincing evidence to suggest that live vaccines may be more effective. Panse *et al.* (1964) showed that infant rabbits, in which experimental cholera can be produced, were passively immunized against the disease by intraperitoneal administration of antisera derived from rabbits immunized with live vibrios. In contrast, antisera obtained with heat-killed and formalin-killed suspensions of the organism were ineffective. This observation was further supported by the finding that the offspring of female rabbits immunized with live vibrios were resistant to experimental infections, whereas female rabbits immunized with killed suspensions of the organism gave birth to susceptible infants (Panse & Dutta, 1964). Feeley (1965) made similar observations and showed that live vibrio antisera, administered intraperitoneally, protected infant rabbits against oral infection with a highly virulent strain of *V. cholerae*. He also observed that the protective antibodies appeared in the later stages of a multiple immunization schedule in rabbits, which synchronised with the preponderance of agglutinin activity in 7S immunoglobulins over that detectable in the 19S factor, the former potentially capable of diffusing more rapidly into the extra-vascular space. In this study as well as in those of Finkelstein (1965*a*) it was emphasized that the protective antibody (antibacterial or antitoxic) should cross the intestinal barrier and appear free in the gut for effective immunity.

The titre of circulating antibody (agglutinating and vibriocidal) seems to bear no relationship to immunity in man, as cases of cholera in the acute phase have shown high antibody titres (Mukerjee, 1963; Finkelstein, Powell, Woodrow & Krevans, 1965). This, considered with the possibility that the effective site of immunity is the intestinal tract, should restore faith in local immune mechanisms, probably mediated by coproantibody (Burrows *et al.* 1947). Freter (1962) and Freter & Gangarosa (1963), using refined methods for estimating coproantibody, showed in human volunteers that these are produced with killed cultures administered orally with a greater regularity than is observed after parenteral immunization. For induction and maintenance of a strong coproantibody response, repeated doses of oral vaccine were required. It was suggested, however, that for reducing

Table 7. *Provisional results obtained in controlled field trials of cholera vaccines*

(Data reproduced from Cvjetanovic (1966), with the permission of the World Health Organization.)

Place, year (project reference)	Vaccines	Strains used	Method of preparation	Side reactions	Maximum efficacy (%)	Duration of immunity	Predominant epidemic strain
Phillipines, 1964–65* (WHO-Phillipines-Japan)	1. Agar-grown *V. cholerae* (fluid)	Ogawa 41† Inaba 35A3†	Phenol	±	63	4 months	El Tor vibrio, Ogawa
	2. Agar-grown El Tor vibrio (fluid)	Ogawa, local Inaba, local	Phenol	±	63	4(–6§) months	
	3. Agar-grown *V. cholerae*, oil adjuvant	Ogawa 41† Inaba 35A3†	Formalin	Very strong	67	More than 1 year	
Calcutta, 1964‡ (WHO-ICMR)	1. Agar-grown *V. cholerae*, freeze-dried	Ogawa 41† Inaba 35A3†	Formalin	±	55§	3 months§	El Tor vibrio, Ogawa
	2. Agar-grown *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	33§	?	
	3. Agar-grown *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	20§	?	
	4. Fluid cultured *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	0§	—	
Calcutta, 1965 (WHO-ICMR)	1. Agar-grown *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	40§	Less than 6 months	El Tor vibrio, Ogawa
	2. Fluid cultured *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	6§	Less than 6 months	
Dacca, 1963–65** (Pakistan-SEATO)	1. Agar-grown *V. cholerae* (fluid) with high antigen content	Ogawa 41†	Heat-killed	Strong	79	More than 1·5 years	*V. cholerae* Inaba

(fluid) indicates the vaccines prepared according to the Requirements for Biological Substances (*WHO techn. Rep. Ser.* 1959, No. 179).

± Uncertain or weak and small reactions.

* Phillippines Cholera Committee (1965). *Bull. Wld Hlth Org.* **32**, 603.

† Reference strains used for mouse protection tests by United States NIH.

‡ Taneja, B. L. (1965). *Proceedings of the Cholera Research Symposium*, p. 373. Washington, D.C.; United States Government Printing Office.

§ Statistically no significance as compared with control group (95% confidence limit).

** Oseasohn, R. O., Benenson, A. S. & Fahimuddin, Md. (1965). *Proceedings of the Cholera Research Symposium*, p. 362. Washington, D.C.: United States Government Printing Office.

the doses of oral vaccine attenuated strains capable of extensive proliferation in the gut may be employed (Freter, 1965). Alternatively, it was proposed that parenteral vaccine may be used to induce coproantibody production followed by spaced doses of oral vaccine to maintain their production.

The ideal cholera vaccine may be one that will be effective in inhibiting the proliferation of the pathogen in the gut, thereby suppressing the release of the choleragenic toxin. The point at issue is whether a suitable vaccine can be discovered which, when administered parenterally, will result in the release of protective antibodies in the lumen of the gut or whether their synthesis can only be achieved by a local antigenic stimulus as by oral vaccines. The success achieved by Haffkine with live vaccines administered parenterally in man (for review, see Cvjetanovic, 1965), as well as the experiments on passive immunization with live vibrio antisera referred to earlier, should justify further research with parenterally administered live vaccines in man, side by side with similar studies with oral live vaccines. The effective route of immunization and the choice of vaccine will be resolved only when the nature of the protective antibodies and means of detecting them are understood.

It is imperative that live cholera vaccines, intended for trial in man, should be stable attenuated strains. The findings recorded in this paper show that such mutants of *V. cholerae* may be isolated in the laboratory and with mutational techniques may be marked for specific nutritional requirements and antibiotic resistance which would permit their easy identification and quick differentiation from wild type vibrio strains. In the absence of such markers, it would be difficult to identify fluctuations in virulence of the attenuated strains occurring either in course of time or during oral vaccine trials in man. The range of markers is capable of further extension which will be very useful especially if they are in contrast to specific fermentations and phage sensitivities generally identified in *V. cholerae*.

SUMMARY

This paper describes the study of a mutant of *Vibrio cholerae* which is shown to be attenuated by its avirulence to mouse, rabbit and chick embryo. This mutant character is stable, and the avirulence of this strain probably results from its inability to multiply actively in the tissues of the infected animals. The need for the study of such attenuated strains as live vaccines in man is discussed, and certain aspects of immunity to cholera are reviewed.

This work was supported by grants from the Indian Council of Medical Research and the World Health Organization. Dr K. Bhaskaran wishes to express his appreciation to Dr B. Cvjetanovic (Chief, Bacterial Diseases, World Health Organization, Geneva) for many stimulating discussions on the subject of cholera vaccines and controlled field trials. He is also grateful to Dr F. Jacob (Pasteur Institute, Paris) for technical hints on the use of N-methyl-N′-nitro-N-nitrosoguanidine for mutant isolations in bacteria. The authors are very grateful to Dr M. L. Dhar (Director, Central Drug Research Institute, Lucknow) for his interest and support.

REFERENCES

BARUA, D. & MUKHERJEE, A. C. (1963). Direct bacterial haemagglutination test for differentiating *El Tor* vibrios from *V. cholerae*. *Bull. Calcutta Sch. trop. Med. Hyg.* **11**, 85.

BESREDKA, A. (1927). *Local Immunization*, Baltimore: Williams and Wilkins Co.

BHASKARAN, K. (1960). Recombination of characters between mutant stocks of *Vibrio cholerae*, strain 162. *J. gen. Microbiol.* **23**, 47.

BHASKARAN, K. (1964). Segregation of genetic factors during recombination in *Vibrio cholerae*, strain 162. *Bull. Wld Hlth Org.* **30**, 845.

BHASKARAN, K. & SINHA, V. B. (1966). Attenuation of virulence in *Vibrio cholerae*. *WHO/Cholera Information* 66.6, p. 13.

BHASKARAN, K., SINHA, V. B. & IYER, S. S. (1966). Study of mutations in *Vibrio cholerae* and *Vibrio el Tor*. *WHO/Cholera Information*/66.6, p. 5.

BURROWS, W. (1965). Cholera vibrio toxicities. *Proceedings of the Cholera Research Symposium*, p. 120. Washington, D.C.: United States Government Printing Office.

BURROWS, W., ELLIOTT, M. E. & HAVENS, I. (1947). Studies on immunity to Asiatic Cholera. IV. The excretion of coproantibody in experimental enteric Cholera in the guinea-pig. *J. infect. Dis.* **81**, 261.

CVJETANOVIC, B. (1965). Earlier field studies of the effectiveness of Cholera vaccines. *Proceedings of the Cholera Research Symposium*, p. 355. Washington, D.C.: United States Government Printing Office.

CVJETANOVIC, B. (1966). WHO activities in the field of cholera. In *Training Course on Cholera WHO/Cholera/*66·5, Calcutta, 7–31 March 1966. Geneva: World Health Organization.

DE, S. N. & CHATTERJI, D. N. (1953). An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J. Path. Bact.* **66**, 559.

DE, S. N., GHOSE, M. L. & CHANDRA, J. (1962). Further observation on Cholera enterotoxin. *Trans. T. Soc. trop. Med. Hyg.* **56**, 241.

DUTTA, N. K. & HABBU, M. K. (1955). Experimental Cholera in infant rabbits: a method for chemotherapeutic investigation. *Br. J. Pharmac. Chemother.* **10**, 153.

FEELEY, J. C. (1965). Passive protective activity of antisera in infant rabbits infected orally with *Vibrio cholerae*. *Proceedings of the Cholera Research Symposium*, p. 231. Washington, D.C.: United States Government Printing Office.

FINKELSTEIN, R. A. (1964). Virulence of El Tor vibrios for chick embryos. *Nature, Lond.* **202**, 609.

FINKELSTEIN, R. A. (1965*a*). Immunological aspects of experimental Cholera. *Proceedings of the Cholera Research Symposium*, p. 58. Washington, D.C.: United States Government Printing Office.

FINKELSTEIN, R. A. (1965*b*). Observations on the nature and mode of action of the choleragenic products of cholera vibrios. *Proceedings of the Cholera Research Symposium*, p. 264. Washington, D.C.: United States Government Printing Office.

FINKELSTEIN, R. A., ATTHASAMPUNNA, P., CHULASAMAYA, M. & CHARUNMETHEE, P. (1966). Pathogenesis of experimental cholera: biologic activities of purified procholeragen A. *J. Immun.* **96**, 440.

FINKELSTEIN, R. A., NORRIS, T. H. & DUTTA, N. K. (1964). Pathogenesis of experimental cholera in infant rabbits. I. Observations on the intra-intestinal infection and experimental cholera produced with cell-free products. *J. infect Dis.* **114**, 203.

FINKELSTEIN, R. A., POWELL, C. J., WOODROW, J. C. & KREVANS, J. R. (1965). Serological responses in man to a single small dose of cholera vaccine with special reference to the lack of influence of ABO blood groups on natural antibody or immunological responsiveness. *Bull. Johns Hopkins Hosp.* **116**, 152.

FINKELSTEIN, R. A. & RAMM, G. M. (1962). Effect of age on susceptibility to experimental cholera in embryonated eggs. *J. infect. Dis.* **111**, 239.

FINKELSTEIN, R. A., SOBOCINSKI, P. Z., ATTHASAMPUNNA, P. & CHARUNMETHEE, P. (1966). Pathogenesis of experimental cholera: identification of choleragen (procholeragen A) by disc immunoelectrophoresis and its differentiation from cholera mucinase. *J. immun.* **97**, 25.

FRETER, R. (1956). Coproantibody and bacterial antagonism as protective factors in experimental cholera. *J. exp. Med.* **104**, 419.

FRETER, R. (1962). Detection of coproantibody and its formation after parenteral and oral immunization of human volunteers. *J. infect. Dis.* **111**, 37.

FRETER, R. (1965). Coproantibody and oral vaccines. *Proceedings of the Cholera Research Symposium*, p. 222. Washington, D.C.: United States Government Printing Office.

FRETER, R. & GANGAROSA, E. J. (1963). Oral immunization and production of coproantibody in human volunteers. *J. Immun.* **91**, 724.

GARDNER, A. D., & VENKATRAMAN, K. V. (1935). The antigens of the cholera group of vibrios. *J. Hyg. Camb.* **35**, 262.

GARDNER, E. W., LYLES, S. T., LANKFORD, C. E. & HAGENS, S. J. (1963). A comparison of the virulence of *Vibrio cholerae* strains for the embryonated egg. *J. infect. Dis.* **112**, 264.

GRIFFITTS, J. J. (1942). The use of mucin in experimental infections of mice with *Vibrio cholerae*. *Publ. Hlth Rep., Wash.* **57**, 707.

HEIBERG, B. (1935). *On the classification of* Vibrio cholerae *and Cholera-like vibrios.* Copenhagen: Arnold Busck.

MUKERJEE, S. (1961). Diagnostic uses of cholera bacteriophages. *J. Hyg., Camb.* **59**, 109.

MUKERJEE, S. (1963). Preliminary studies on the development of a live oral vaccine for anti-cholera immunization. *Bull. Wld Hlth Org.* **29**, 753.

OZA, N. B. & DUTTA, N. K. (1963). Experimental cholera produced by toxin prepared by ultrasonic disintegration of *V. comma*. *J. Bact.* **85**, 497.

PANSE, M. V., & DUTTA, N. K. (1964). Cholera vaccines and placental transmission of antibodies. *J. Immun.* **93**, 243.

PANSE, M. V., JHALA, H. I. & DUTTA, N. K. (1964). Passive immunity in experimental cholera. *J. infect. Dis.* **114**, 26.

PESIGAN, T. P. (1965). Cholera and El Tor vaccines used in the controlled field trials in the Philippines by the joint Philippine-Japan-WHO Cholera Committee. *Proceedings of the Cholera Research Symposium*, p. 171. Washington, D.C.: United States Government Printing Office.

PITTMAN, M. & FEELEY, J. C. (1965). Laboratory assay of Cholera vaccine potency. *Proceedings of the Cholera Research Symposium*, p. 163. Washington, D.C.: United States Government Printing Office.

REED, L. J. & MUENCH, H. (1938). A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493.

SHRIVASTAVA, D. L. & WHITE, P. B. (1947). Note of the relationship of the so-called Ogawa and Inaba types of *Vibrio cholerae*. *Indian J. med. Res.* **36**, 409.

EXPLANATION OF PLATE

Two ileal loops of an adult rabbit, showing the difference between the effects of virulent and attenuated strains of *Vibrio cholerae* introduced into the lumen of the gut.

K. BHASKARAN AND V. B. SINHA (*Facing p*. 148)

J. Hyg., Camb. (1967), **65**, 149
Printed in Great Britain

Human infection with *Salmonella choleraesuis* in Hong Kong

By C. T. HUANG and C. B. LO
Department of Pathology and Bacteriology, University of Hong Kong

(*Received* 21 *November* 1966)

INTRODUCTION

Human infection with *Salmonella choleraesuis* has been reported in China repeatedly. In an investigation of the salmonella serotypes isolated from clinical specimens in Hong Kong, Huang & Chan-Teoh (1964) found that *S. choleraesuis* was second to *S. typhi* in prevalence amongst hospital patients. This prevalence of *S. choleraesuis* infection is most probably the result of similarity in cooking, eating and related customs here and in China. The report of Huang & Chan-Teoh (1964) is extended in the present paper to include another nine strains of *S. choleraesuis* that have been isolated in the same laboratory subsequently. We have re-investigated and compared the cultural characters, drug sensitivities and H_2S production of a total of 49 strains of *S. choleraesuis* isolated from 47 patients between July 1953 and June 1966 and tabulated the clinical presentation of the cases.

MATERIALS AND METHODS

Isolation and identification from clinical materials

The methods used in a previous study (Huang & Chan-Teoh, 1964) were applied also to the new cases reported here. Failure to ferment trehalose and arabinose were the criteria taken to discriminate *S. choleraesuis* from the serologically related serotypes *S. paratyphi C*, *S. decatur* and *S. typhisuis*. Differentiation of *S. choleraesuis* into diphasic, H_2S-negative American variety and monophasic, H_2S-positive Kunzendorf variety was carried out by using infusion-agar slants and TSI-agar slants (Edwards & Ewing, 1955) and by slide agglutination with specific (c) and non-specific (1, 5) sera obtained from the Wellcome Research Laboratories, England. To test for H_2S production, both the butt and the slope of the TSI-agar were inoculated with the culture. A strip of filter-paper soaked in 10 % solution of lead-acetate and then dried was held in place in the inner side of the test-tubes containing the media by cotton plugs. The cultures were incubated at 37° C.

Drug sensitivity tests

The usual disk method was employed. Oxoid S.T. agar and Oxoid multodisks containing respectively streptomycin, tetracycline, chloramphenicol, sulphafurazole, polymyxin B and nitrofurantoin were used. In addition, disks containing kanamycin and neomycin prepared in this laboratory were included. Later, when disks became available, some of the old stock strains and the freshly isolated strains were tested against nalidixic acid, ampicillin, gabbromicina, cephaloridine and

colistin. The concentrations of the drugs and the numbers of strains tested against each drug are given in Table 5. The cultures were incubated at 37° C. and read for inhibitory zones of growth 18 hr. afterwards.

RESULTS

Age incidence and sex ratio

The age incidence and sex ratio is indicated in Table 1. The youngest patient was a male 7 months old who had an abscess of the right thigh and the organism was isolated from the pus. The oldest patient was a woman of 81 years and the organism was recovered from the pus of a breast abscess. The incidence corresponds with the findings of Saphra & Wassermann (1954).

Table 1. *Age incidence*

	Nos. of patients from whom *S. choleraesuis* was isolated	
Age group	Male	Female
Under 1	2	2
1–10	14	4
11–20	1	0
21–30	4	1
31–40	6	1
41–50	5	0
51–60	0	1
Over 60	2	4
Total	34	13

Frequency of occurrence and the ratio of incidence to other salmonella serotypes

Table 2 displays the comparative frequency of *S. choleraesuis* in clinical specimens examined in this laboratory in 13 years. Its occurrence fluctuated from year to year in a manner not related to the total numbers of specimens examined. Only 49 strains were isolated from more than 100,000 specimens, so that *S. choleraesuis* was, in general, not encountered commonly. But among salmonella serotypes isolated from hospital patients it ranked second to *S. typhi* in prevalence (Huang & Chan-Teoh, 1964).

Correlation of clinical manifestations and types of specimen

Table 3 correlates the clinical manifestations and the specimens from which *S. choleraesuis* was isolated. The clinical syndromes are arranged according to Saphra & Wassermann (1954). The organism was isolated most frequently from blood cultures and was recovered more often from pus and aspirates than from faeces. Rarely it was isolated from cerebrospinal fluid, urine and sputum. The frequent isolation of the organism from blood cultures reveals the septicaemic tendency of the infection in man. Only five faecal specimens were positive. Apparently transient gastro-enteritis caused by this organism was rare (Saphra &

Table 2. *Ratio of* S. choleraesuis *to other salmonella serotypes*

Year	Total no. of specimens examined	No. of strains of salmonella serotypes (including *S. typhi*)	No. of *S. choleraesuis*	Percentage of *S. choleraesuis* to total salmonella serotypes
1953–54	1,008	9	0	—
1954–55	1,522	19	0	—
1955–56	1,585	17	4	23·5
1956–57	2,325	20	4	20·0
1957–58	3,027	34	3	8·8
1958–59	3,091	15	3	20·0
1959–60	6,637	34	2	5·9
1960–61	9,372	51	6	11·8
1961–62	10,807	55	3	5·5
1962–63	13,651	76	13	17·1
1963–64	14,947	51	5	9·8
1964–65	15,373	47	3	6·4
1965–66	21,945	49	3	6·1
Total	105,290	477	49	10·3

Table 3. *Correlation of clinical manifestations and specimens with positive cultures from different sources in* 47 *patients*

	No. of strains of *S. choleraesuis* isolated						
Clinical manifestations	Blood	Sputum	Urine	Stool	C.S.F.	Pus and aspirates	Total
1. Gastroenteritis	—	—	—	3	—	—	3
2. Fever, septic type	6	—	—	—	—	—	6
3. Fever, typhoid type	4	—	—	1	—	—	5
4. Bronchopneumonia	4	1	—	—	—	—	5
5. Localized suppurative process							
Peritonitis	1	—	—	—	—	—	1
Lung abscess	1	—	—	—	—	—	1
Liver abscess	2	—	—	—	—	3	5
Perinephric abscess	—	—	—	—	—	1	1
Brain abscess	—	—	—	—	—	1	1
Meningitis	1	—	—	—	2	—	3
Osteomyelitis	1	—	—	—	—	2	3
Cervical abscess	—	—	—	—	—	2	2
Parotid abscess	—	—	—	—	—	1	1
Breast abscess	—	—	—	—	—	1	1
Abscess of lower limbs	—	—	—	—	—	3	3
6. Chronic debilitating diseases							
Carcinoma of liver	—	—	2	1	—	—	3
Monocytic leukaemia	1	—	—	—	—	—	1
Chronic lymphocytic leukaemia	2	—	—	—	—	—	2
Haemolytic anaemia	1	—	—	—	—	—	1
Syphilitic aneurysm	—	1	—	—	—	—	1
Total	24	2	2	5	2	14	49

Wassermann, 1954); or perhaps patients with this kind of infection were rarely admitted to hospital because of the mildness of their symptoms. Table 3 also lists the non-specific clinical syndromes complicated by *S. choleraesuis*. Febrile infections of either typhoid or septicaemic types and localized suppurative infections were common. The organism was isolated many times in chronic debilitating diseases such as carcinoma of the liver, leukaemia, haemolytic anaemia and syphilitic aneurysm. Among all the cases reported here, only one death ascribable to the infection took place in hospital and is known to us. It was a case of hepatosplenomegaly with haemolysis complicated by *S. choleraesuis* infection. The organism was isolated twice from blood cultures before death.

Table 4. *Biochemical reactions of* S. choleraesuis *isolated*

	No. of strains tested	% of positive strains
Motility	49	100
Glucose	49	100
Lactose	49	0
Mannite	49	100
Sucrose	49	0
Salicin	49	0
Dulcitol	40	50
Xylose	40	100
Rhamnose	40	100
Inositol	40	0
Arabinose	49	0
Trehalose	49	0
D-Tartrate	22	100
H_2S production		
(*a*) with lead acetate paper	49	6*
(*b*) in butt of TSI agar	49	4†
Indole	49	0
M.R.	40	100
V.P.	40	0
Citrate	40	100
Urease	40	0
Nitrate	40	100
Gas production in fermented sugar	49	86

* Two diphasic strains, one monophasic strain.

† One diphasic and one monophasic strain.

Biochemical reactions of the strains isolated

Table 4 presents the biochemical reactions of the strains isolated. These agree with those described by others (Kauffmann, 1954; Edwards & Ewing, 1955; Wilson & Miles, 1964). Half of the strains fermented dulcitol within 24 hr. incubation. Seven anaerogenic strains were encountered; two were reported previously (Huang & Chan-Teoh, 1964) and two were recently isolated. These four were anaerogenic from primary isolation. The other three were aerogenic on first isolation however, and anaerogenic substrains emerged only when stock cultures were re-tested. Gas production from sugar fermentation is thus not a constant character

of *S. choleraesuis*. It varied not only from strain to strain but also in substrains of a gas-producing strain.

Only one monophasic, H_2S-positive Kunzendorf variety was found. This strain blackened not only the lead-acetate filter-paper strips suspended over either infusion- or TSI-agar, but also the butt of the latter medium. According to Edwards & Ewing (1955), TSI-agar is less sensitive for testing H_2S production than lead-acetate paper strip. Out of 48 diphasic strains, two gave definite blackening of the lead-acetate paper strips and one of these two blackened the butt of the TSI-agar. The blackening of the paper strips placed over the culture in the TSI-agar was more intense than that of the strips over infusion-agar. Slide agglutinations with monophasic specific (c) and non-specific (1, 5) sera were repeatedly positive in both. They were, therefore, H_2S-positive diphasic strains. Subsequent tests by Craigie tube technique to obtain monophasic substrains were repeatedly H_2S-positive, trehalose and arabinose negative. When 0·0000001 ml. of a 24 hr. broth culture of these substrains was injected subcutaneously into rabbits weighing about 2 kg., the animals died within 10 days. On the other hand, 0·1 ml. of a 24 hr. broth culture of a strain of *S. paratyphi C* injected similarly failed to kill the rabbits injected. The virulence of these monophasic substrains of *S. choleraesuis* for rabbits is accordingly the same as that reported by Tenbroeck, Li & Yü (1931) in Kunzendorf strains.

Table 5. *Results of drug sensitivity tests*

Drug employed	No. of strains tested	% of sensitive strains
Streptomycin, 10 μg.	30	0
Tetracycline, 10 μg.	30	100
Chloramphenicol, 10 μg.	30	100
Nitrofurantoin, 50 μg.	30	100
Polymyxin B, 100 units	30	100
Sulphafurazole, 100 μg.	30	43
Ampicillin, 25 μg.	30	100
Neomycin, 10 μg.	30	100
Kanamycin, 10 μg.	30	100
Nalidixic acid, 30 μg.	22	100
Gabbromicina, 5 μg. (aminosidine sulphate)	22	100
Cephaloridine, 25 μg.	22	100
Colistin, 5 μg.	22	100

Drug sensitivities

The results of drug sensitivity tests are presented in Table 5. All strains were insensitive to streptomycin. Oxoid S.T. agar which has a reduced salt concentration (0·3 % NaCl) was used, and the insensitivity to streptomycin is an inherent property of *S. choleraesuis* and is not due, in our study, to the influence of the salt content of the medium indicated by Barber & Garrod (1963). The organism was sensitive to kanamycin and neomycin and so there was no cross-resistance to drugs related to streptomycin. *S. choleraesuis* was sensitive to most of the drugs tested including the more recently introduced ones, such as nalidixic acid, ampicillin, gabbromicina, cephaloridine and colistin.

GEOGRAPHICAL DISTRIBUTION OF *SALMONELLA CHOLERAESUIS* IN CHINA AND OTHER EAST ASIAN COUNTRIES

China

Human infection due to *S. choleraesuis* has been reported in many parts of the world since Salmon & Smith described the organism in 1885 (Wilson & Miles, 1964). That it is endemic in China is revealed in Tables 6–8. Table 6 presents the incidence and distribution of the various salmonellas including *S. choleraesuis* that have been reported by different workers in China. In Table 7 the reports of *S. choleraesuis* in China from 1927 to 1959 are arranged chronologically, and Table 8 is an excerpt of the wartime data presented by Japanese workers. The earliest report of human infection due to this organism was that of Hicks & Robertson (1927) from Shanghai. Following that many reports appeared in the literature (Tables 7 and 8). However, since 1955 most of the reports were published in the Chinese language and are inaccessible to many workers. These reports are, therefore, briefly reviewed in the following paragraphs.

In addition to the report of Lu & Yeh (1955), which was written in English and in which 45 cases of *S. choleraesuis* infection examined in Shengyang, Liaotung during 1951–52 were described, two more reports of *S. choleraesuis* infection appeared in the Chinese literature in the same year. Jen & Pan (1955) described two cases which occurred in Peking, and were successfully treated with chloromycetin. Ku, Li & Chang (1955) observed 2 cases with three deaths in Wuhan, Central China. Over half of their cases were the typhoid-septicaemia type and the organism was isolated from blood, bone-marrow and faeces of all patients and from the peritoneal fluid of one. Seven of the 26 cases were in children under 2 years. In the following year, Liu (1956) described 54 cases in children all with positive blood cultures occurring in the Kweilin area of Kwangsi Province. Eight of the 54 children died. He considered that his cases were associated with an epidemic of hog-cholera then prevailing in the region. He noted that farmers slaughtered the sick animals and sold the meat to the market at a reduced price; three-fifths of the pigs he examined carried *S. choleraesuis* in their blood.

Several reports of *S. choleraesuis* infection appeared concurrently in the Chinese medical literature in 1957. Liao (1957) briefly listed the incidence of *S. choleraesuis* infection in China and described 113 Kunzendorf strains examined by him in Kweiyang, Kweichow Province; 108 of these strains were isolated from human blood cultures. Another four were isolated from pigs and one from the shell of an egg. Liao stated that for 2 years *S. choleraesuis* headed the list of salmonellas in Kweichow Province accounting for 37·6 % as against 33·8 % for *S. typhi*. Hsu & Chang (1957) reported that *S. choleraesuis* formed 42·6 % of the total number of salmonellas, other than *S. typhi*, occurring in Hunan Province between 1953 and 1956, and that the percentage had increased over this period in which laboratory methods of diagnosis had remained unchanged. They concluded that the disease was assuming considerable epidemiological importance. They presented the clinical symptoms of 23 cases with three deaths. The true incidence might have been found to be higher if the patients were not indiscriminately

Table 6. *Incidence of salmonellosis in man in China*

Authors ...	Wu & Zia	Pasteur* Institute	Chih & Chien	Lu & Yeh	Ku *et al.*	Fu *et al.*	Hsu & Chang	Liao	Fu *et al.*†
Year published...	1935	1949	1949	1955	1955	1957	1957	1957	1957
Region ...	Peking	Shanghai	Chungking	Liaotung	Wuhan	Chengtu	Changsha	Kweiyang	Shanghai
S. paratyphi A	65‡ (9·4)	64 (10·8)	10 (3·2)	3 (2·0)	10 (6·6)	5 (3·0)	11 (6·7)	11 (3·8)	59
S. paratyphi B	22 (3·2)	47 (7·9)	5 (1·6)	9 (5·7)	4 (2·6)	2 (1·2)	15 (9·2)	64 (22·3)	29
S. typhi-murium	—	3 (0·5)	1 (0·3)	—	—	11 (6·6)	—	1 (0·4)	5
S. derby	—	5 (0·9)	—	—	—	7 (4·2)	—	3 (1·1)	3
S. paratyphi C	2 (0·3)	12 (1·9)	—	29 (18·4)	5 (3·3)	—	4 (2·5)	—	13
S. choleraesuis									
var. *american*	—	19 (3·2)	—	41 (26·1)	26 (17·1)	14 (8·3)	23 (14·1)	—	23
var. *kunzendorf*	21 (3·0)	—	3 (0·9)	4 (2·5)	—	7 (4·2)	—	108 (37·6)	2
S. thompson	—	5 (0·9)	—	—	—	—	—	—	2
S. potsdam	—	1 (0·2)	—	—	—	—	—	—	—
S. newport	—	2 (0·3)	—	—	—	—	—	1 (0·4)	1
S. typhi	581 (84·1)	403 (68·1)	297 (93·7)	69 (44·0)	107 (70·4)	102 (61·1)	109 (66·9)	97 (33·8)	—
S. sendai	—	1 (0·2)	—	—	—	—	—	—	2
S. dublin	—	—	—	—	—	—	—	1 (0·4)	7
S. enteritidis	—	7 (1·1)	1 (0·3)	—	—	—	1 (0·6)	1 (0·4)	—
S. blegdam	—	16 (2·6)	—	—	—	—	—	—	—
S. gallinarum	—	—	—	—	—	3 (1·8)	—	—	—
S. london	—	1 (0·2)	—	—	—	—	—	—	—
S. anutum	—	5 (0·9)	—	—	—	15 (9·0)	—	—	—
S. seftenberg	—	—	—	2 (1·3)	—	—	—	—	—
S. aberdeen	—	1 (0·2)	—	—	—	—	—	—	—
Not defined	—	—	—	—	—	1 (0·6)	—	—	1
Total	691	592	317	157	152	167	163	287	147

* Quoted from Le Minor (1964).
† *S. typhi* excluded.
‡ Figures indicate numbers of strains.
Figures in parentheses indicate percentage incidence of serotypes in each report.

treated with antibiotics immediately after admission to hospital. Fu, Lin, Chou, Chen, Pan & Ch'iu (1957) reported that 21 out of 65 cases of salmonellosis in Chengtu in West China were due to *S. choleraesuis*. They noted that the incidence of this organism in Chengtu was just as high as it was in other parts of China. Wang, Peng, Hsieh & Tang (1957) described a fatal case of *S. choleraesuis* septic-

Table 7. *Human* S. choleraesuis *infections reported in China between* 1927 *and* 1959

Authors	Year published	Region	*S. choleraesuis* American variety	*S. choleraesuis* Kunzendorf variety
Hicks & Robertson	1927	Shanghai	1	—
Tenbroeck *et al.*	1931	Peking	—	3
Wu & Zia	1935	Peking	—	18
Huang *et al.*	1937	Peking	3	—
Ling *et al.*	1941	Shanghai	2	—
Raynal & Fournier	1947	Shanghai	2	—
Annual Report (Pasteur Institute)*	1949	Shanghai	19	—
Fournier & Ma	1949	Shanghai	—	1
Chih & Chien	1949	Chungking	—	3
Wu & Fournier	1950	Shanghai	—	1
Lu & Yeh	1955	Liaotung	41	4
Jen & Pan	1955	Peking	2	—
Ku *et al.*	1955	Wuhan	26	—
Liu	1956	Kweilin	54	—
Liao	1957	Kweiyang	—	108
Hsu & Chang	1957	Changsha	23	—
Fu *et al.*	1957	Chengtu	14	7
Wang *et al.*	1957	Ya-an	1	—
Fu *et al.*	1957	Shanghai	23	2
Tsou	1959	Wuhan	30	—
Totals			241	147

* Quoted from Le Minor, 1964.

Table 8. *Distribution of* Salmonella choleraesuis *isolations in war-time China, based on Y. Aoki* (1964)

Authors	Year published	Region	No. of *S. choleraesuis* strains isolated
Kubota	1939	Canton	2
Shóji	1942	Wuhu	80
Ogawa-Satō	1943	Tsingtao	4
Nagaki	1943	Honan Prov.	13
Umemoto	1943	N. China	13
Komori	1943	S. China	12
Hiroki	1949	Manchuria	59
		N. China	54
Hamano	1951, 1956	Lushun-Dairen	10
		Manchuria	203
		N.E. & C. China	131
		Taiwan	7
Total			588

aemia complicating staphylococcal osteomyelitis of the femur from Ya-an city which lies on the western border of Szechwan Province west of Chengtu. The patient, who tended pigs for her family, developed osteomyelitis of the right femur involving the right hip joint. *Staphylococcus aureus* was isolated twice from discharge from the bone lesions; blood cultures, however, yielded *S. choleraesuis* which was agglutinated by the patient's serum.

Also in 1957, Fu, Wu, Lu & Ch'en (1957) recorded that 25 out of 147 cases of non-typhoid salmonellosis (17·0 %) in Shanghai were due to *S. choleraesuis*. Two of these 25 belonged to the Kunzendorf variety. Eight (34·8 %) of the patients were children, including a 10-day-old baby, which as far as we are aware, is the youngest patient on record. Four fatal cases due to the American variety were all in children; three were infants and one was a 6-year-old girl. It is interesting to note that all outbreaks reported in 1957 occurred in places quite remote from each other.

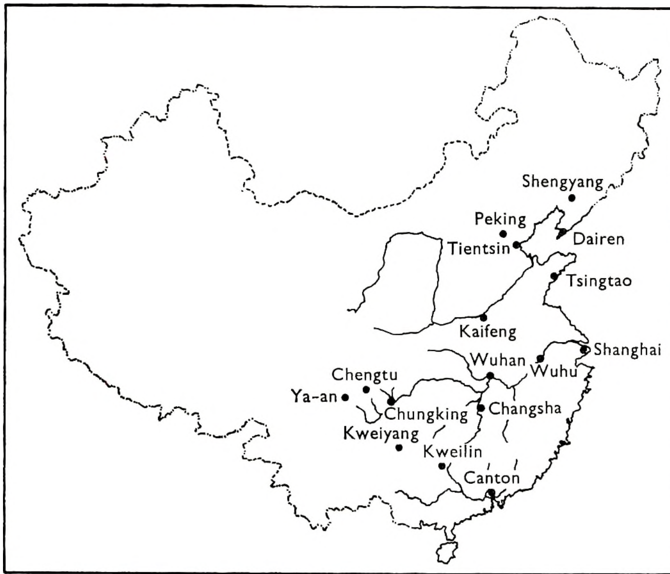


Fig. 1. Distribution of *S. choleraesuis* infection in the continent of China. Locations indicated are based on the reports presented in Tables 7 and 8.

More recently, Tsou (1959) analysed the clinical manifestation of 30 cases of *S. choleraesuis* infection occurring in Wuhan, Wupeh Province. Most of the patients were under 3 years. All, except one case, were of the primary typhoid-septicaemia type and no death was recorded.

This review indicates clearly that *S. choleraesuis* infection is distributed widely in China (Fig. 1). Its reported extent reaches from the North Eastern Provinces (Manchuria) down to Canton in Kwangtung Province which lies in the south and in the vicinity of Hong Kong, and also from Shanghai on the eastern coast to as far west as Ya-an in Szechwan Province. Aoki (1964) emphasized that there was a great difference between the incidence of salmonellosis in peace and in war, but to judge from its frequency before, during and after the Second World War, one may concede that *S. choleraesuis* is endemic in China.

East Asian countries

Although *S. choleraesuis* infection is prevalent in China, it is relatively rare in the surrounding East Asian territories. Sakazaki & Nakaya (1964), in a systematic epidemiological and ecological study of salmonellas between 1940 and 1963 in

Table 9. *Comparison of the percentage of* S. choleraesuis *infection in man in different regions of the world*

	Authors	Places	Date of isolation	Total no. of strains of salmonella serotypes (excluding *S. typhi*)	Total no. of strains of *S. chol-leraesuis*	Percentage of *S. chol-eraesuis* to salmonella serotypes	Remarks
A	Silberstein & Gerichter	Israel	1949–62	17,335	0	0	P. 340, Table 4
A	Van Oye	Central Africa	1946–60	1,557	0	0	P. 356, Table 1
A	Le Minor	Madagascar	1949–60	125	4	3·2	P. 367, Table 1
A	Le Minor	Nigeria	1955–59	456	0	0	P. 368, Table 2
A	Le Minor	Senegal	1950–62	216	8	3·7	P. 376, Table 5
A	Bynoe & Yurack	Canada	1953–62	12,314	30	0·3	P. 400, Table 2
A	Galton, Steele & Newell	United States	1934–63	30,589	746	2·4	P. 428, Table 3
A	Olarte & Varela	Mexico	1940–62	1,538	32	2·1	P. 467, Table 24
A	Peluffo	South America	Not given	2,610	86	1·5	P. 494, Table 10
A	Fukumi	Japan	1951–53	119	0	0	P. 516, Table 7
A	Le Minor	Vietnam	1952–61	482	41	8·5	P. 531–4, Tables A, B, C
A	Atkinson	Australia	1950–62	7,182	51	0·7	P. 544, Table 1
B	Various authors	China	1927–57	908	291	32·0	
C	Huang & Chan-Teoh	Hong Kong	1953–63	132	30	30·3	

A. Quoted from Van Oye (1964). Page and table numbers refer to those whence the data derived.
B. Average calculated from data in Table 6.
C. Quoted from Huang & Chan-Teoh's (1964).

Japan, typed 3892 strains. Of these, only 12 strains were *S. choleraesuis* (seven from animals and five from man) and all were isolated before 1950. According to these authors *S. choleraesuis* has not been found in Japan since 1950 and their work was confirmed by Fukumi (1964). Aoki (1964) stated that Hagakawa and his

colleagues had reported in 1944 that only three of 1210 strains of salmonellas (0·25 %) collected by them from Java, Sumatra, Malaya, Thailand, Vietnam, the Philippines and Taiwan during the war were *S. choleraesuis*. The rare occurrence of the organism in the South Pacific region was evidenced by the work of Lindberg & Bayliss (1946) who typed 202 strains of salmonellas isolated between 1944 and 1945 and found only one strain of *S. choleraesuis*. Cheng (1964) investigated the salmonella serotypes from man, fowls and animals in Taiwan; he recovered 39 strains of *S. choleraesuis* from pigs but not from man. In wartime records, however, Hamano (Aoki, 1964) found seven out of 351 (1·99 %) of the total *S. choleraesuis* strains he had studied were isolated in Taiwan (Table 8), but even in those disturbed times the incidence of *S. choleraesuis* in Taiwan was three to ten times less than that in the mainland of China. Nguyen & Fournier (1960), describing a case of liver abscess in South Vietnam, stated it was not unusual to recover this organism from bacterial liver abscess there. Courmes & Bres (1954), on the other hand, found only one strain of *S. choleraesuis* among 173 strains of salmonellas they typed in the same country. According to Le Minor (1964) the percentage of *S. choleraesuis* among all salmonella serotypes in South, Central and North Vietnam were 1·4, 2·6 and 1·7 % respectively. Zimmerman, Cooper & Graber (1952) studied an outbreak of salmonellosis among prisoners of war in Korea and reported that 43 out of 357 strains (12 %) of salmonellas they had isolated were *S. choleraesuis* var. *kunzendorf*. Chun (1964) reviewed salmonella and shigella infections among civilians and military personnel in Korea, and reported that *S. choleraesuis* occurred in 0·4 % of one survey and in 4·7 % of another carried out in 1952–54. But not a single case was seen in a third survey performed in 1961–63 long after the Korean war. Considering the report of Lu & Yeh (1955) of a high incidence in neighbouring Manchuria, we are inclined to think that the incidence reported by Zimmerman *et al.* and in Chun's first two surveys was unusual in Korea and was exceptionally influenced by war-time conditions.

Table 9 summarizes the incidence of *S. choleraesuis* reported in several different areas of the world. It reveals that the percentage of *S. choleraesuis* among all other salmonella serotypes (excluding *S. typhi*) is uniquely high in China and in hospital patients in Hong Kong.

DISCUSSION

In this series, 22 out of 49 strains (45 %) of *S. choleraesuis* were isolated from patients under 10 years of age. This accords with the reports of other Chinese workers (*loc. cit*). Of the 22 strains in children 16 were isolated from blood cultures, three from pus, two from faeces and one from c.s.f. Fevers of septic and typhoid type were encountered more often in children, whereas localized suppurative infections were the frequent clinical manifestations in adults. The low resistance of young children may be due to absence of previous contact and immunity against a potentially septicaemic organism. The relatively frequent occurrence of infection in adults debilitated by chronic organic diseases supports the view that resistance is generally effective, although it may be sapped by a heterologous infection or by neoplastic diseases, etc. (Saphra & Winter, 1957). The low mortality rate of one

detected fatal case in our series, compared with the relatively high death-rate prevailing a decade or two ago, can be attributed to modern therapy. According to Saphra & Winter (1957) the death-rate formerly approached 20·3 %.

In vitro sensitivity tests show a wide choice of inhibitory drugs against *S. choleraesuis*. Because of the fulminant nature of the untreated disease and the irregularity of the clinical picture, early and accurate bacteriological diagnosis is essential. Premature administration of antibiotics before the collection of specimens for bacteriological study defeats the objective and more cases might be discovered if the frequency of the organism was better appreciated and more effort made in its isolation.

It has been suggested that paratyphoid C should be included in the TAB vaccine used in regions where *S. choleraesuis* or group C salmonella infection is prevalent (Wu & Zia, 1935; Saphra & Winter, 1957). There are, however, better ways of obtaining protection against *S. choleraesuis* infection. Smith (1965) showed that a live vaccine made from rough variants of *S. choleraesuis*, though possessing a considerable degree of virulence for mice, could protect the survivors from experimental oral infection. Furthermore, his vaccine also produced an appreciable degree of immunity in pigs. According to Tenbroeck *et al.* (1931), *S. choleraesuis* was much more virulent than *S. paratyphi C* to rabbits. This has been confirmed by us. Whatever type of vaccine against *S. choleraesuis* may be developed in the future, the organism itself should be used in preference to *S. paratyphi C*.

The differentiation of *S. choleraesuis* into two varieties has been criticized. Wilson & Miles (1964) cited Kauffmann's view that the designation 'Kunzendorf variety' should be abolished. The occurrence in our material of the two H_2S positive diphasic strains confirms that the test for H_2S production can no longer be considered valuable for taxonomical classification of *S. choleraesuis* into varieties though it has some use in the epidemiological study of a strain. There is no substantial difference in pathogenicity between the monophasic and diphasic varieties either. The reports of fatal cases by Wu & Zia (1935) and by Fournier & Ma (1949) verified the virulence of the H_2S-positive monophasic variety.

Table 9 shows that the percentage of *S. choleraesuis* among salmonella serotypes (excluding *S. typhi*) in our series (30·3 %) is very close to the average percentage in China (32·1 %). Though our finding does not represent the overall incidence of *S. choleraesuis* in Hong Kong, the strains had been subjected to thorough bacteriological study. The incidence might have been higher if group C salmonella or unidentified salmonella strains reported by other laboratories in Hong Kong were subjected to further typing. It is no coincidence that Hong Kong lies on the edge of the Chinese continent and that over 90 % of the population have the same customs as the people in the mainland of China. Pork makes up a large part of the protein in the diet and clearly plays a part in disseminating the organism. Confirmatory evidence is found in the fact of low incidence in other regions of the world where pork is a less important element in the diet or even not used. In Israel, where pork is excluded from foodstuffs, *S. choleraesuis* infection is evidently completely absent (Table 9).

Pigs are the main carriers of *S. choleraesuis* (Saphra & Wassermann, 1954) and

processed pork or sausages are the usual source of the infection (Hobbs, 1964). It is animals weakened by diseases such as hog-cholera or pneumonia or otherwise stressed that develop septicaemia due to the organism (Cheng, 1964). Pork and pig offal derived from uncontrolled butchery are, therefore, dangerous for human consumption. Even under conditions of controlled butchery in Hong Kong *S. choleraesuis* can be identified occasionally in material from pigs. Where pigs in poor condition are particularly examined, a considerable number are found to carry the organism (Lo, Huang & Chan, to be published). Whether active or not porcine infection is common in animals brought for slaughter here because over 50% have agglutinins against CO and CH in their sera. Pigs are thus a dangerous potential source of human *S. choleraesuis* infection in Hong Kong. Ding, Liu & Chih (1956) reported that the highest rate of isolation of *S. choleraesuis* from sick hogs was from the mesenteric lymph-nodes, and next from the liver; meat rarely gave positive cultures. Smith (1959) also maintained that the rate of isolation from the mesenteric lymph-nodes of healthy pigs was high; he isolated *S. typhimurium* and *S. anatum* more commonly. The liver, kidneys, lungs and intestines of pigs are very often used in the local cuisine of Hong Kong. When such articles of diet are derived from sick animals which have escaped detection and inspection they are certainly dangerous for consumption. Moreover, blood clot from pigs is also eaten by people here. The clot is sliced into small cubes and steamed in rice gruel. The temperature of the gruel may kill organisms on the surface of the clot, but cannot be relied on to kill those within the cubes. Although direct evidence of this mechanism of transmission of *S. choleraesuis* is not available yet, it seems likely that the habit of eating pig offal accounts in part at least for the prevalence of *S. choleraesuis* infection in the local population. The occasional infections reported in dogs (Galton, Scatterday & Hardy, 1952), cats (Huang Chan-Teoh, 1964) and rats (Fournier, 1939) suggest that these animals may play a part in transmission too.

Because *S. choleraesuis* generally attacks only young or debilitated persons, we think that the invasiveness of *S. choleraesuis* is not as high as has often been suggested. Saphra & Winter (1957) maintained that much salmonellosis went untreated, undiagnosed and bacteriologically unidentified and that only the severe parenteral and fatal infections stimulated adequate efforts, for instance when pus, blood, exudates and spinal fluid were examined. This is probably true for *S. choleraesuis* infection and, if so, would provide an explanation for what seems an unexpectedly high fatality rate in the older series. On the other hand, it is important that the infection should not be overlooked in communities like Hong Kong where it ranks as the second commonest of the salmonelloses—for persons with low resistance often succumb to it once they are infected.

SUMMARY

The literature of human infection with *Salmonella choleraesuis* in China was fully reviewed with special reference to those written in Chinese language which are inaccessible to many readers. Compared with other regions of the world the ratio

of *S. choleraesuis* to other salmonella infection in Hong Kong is high and is parallel to that in China. Dietetic habits of the local population apparently contribute to the high incidence. The clinical manifestations of 47 cases were outlined and details of the biochemical reactions and *in vitro* drug sensitivities of these strains were described. Two diphasic H_2S-positive strains were encountered.

The authors are most grateful to Prof. James B. Gibson for his helpful advice and constructive criticism.

REFERENCES

Aoki, Y. (1964). Distribution of salmonella types in East Asia. 1. Distribution of salmonella types in the Chinese continent during the period from 1937 to 1944. *Endem. Dis. Bull. Nagasaki Univ.* **6**, 174.

Barber, M. & Garrod, L. P. (1963). *Antibiotics and Chemotherapy*, p. 340. London: E. and S. Livingstone Ltd.

Cheng, C. M. (1964). Salmonella in Taiwan, especially in animal diseases (Preliminary report). *Endem. Dis. Bull. Nagasaki Univ.* **6**, 148.

Chih, C. S. & Chien, Y. B. (1949). Systemic salmonella infection in man. *Chin. med. J.* **67**, 51.

Chun, D. (1964). A review of salmonella and shigella in Korea. *Endem. Dis. Bull. Nagasaki Univ.* **6**, 125.

Courmes, E. & Bres, P. (1954). Étude bactériologique et épidémiologique des salmonelloses au Sud. Vietnam en 1953. *Bull. Soc. Path. exot.* **47**, 302.

Ding, S. H., Liu, S. Y. & Chih, S. L. (1956). Investigation into carrier conditions of salmonella in fowls and animals. *Shanghai vet. Bull.* **1**, 9 (in Chinese).

Edwards, P. R. & Ewing, W. H. (1955). *Differentiation of Enterobacteriaceae*, p. 9. Minnesota: Burges Publishing Co.

Fournier, J. (1939). Deux cas de septicémie à *Salmonella cholerae-suis* à Changhai. *Bull. Soc. Path. exot.* **32**, 914.

Fournier, J. & Ma, K. T. (1949). Sporadic salmonella infection in Shanghai during summer of 1948. *Chin. med. J.* **67**, 16.

Fu, J. S., Lin, C. C., Chou, P. C., Chen, Y. L., Pan, C. J. & Ch'iu, C. C. (1957). A preliminary report of *Salmonella choleraesuis* infection in Chengtu. *Chin. J. intern. Med.* **5**, 618 (in Chinese).

Fu, W. S., Wu, M. C., Lu, C. C. & Ch'en, N. P. (1957). A clinical report of salmonella infection in Shanghai in 1955. *Nat. med. J. China* **43**, 497 (in Chinese).

Fukumi, H. (1964). Salmonelloses in Japan. In *The World Problem of Salmonellosis*, pp. 507–29. Ed. E. Van Oye. The Hague: Dr. W. Junk.

Galton, M. M., Scatterday, J. E. & Hardy, A. V. (1952). Salmonellosis in dogs. I. Bacteriological, epidemiological and clinical consideration. *J. infect. Dis.* **91**, 1.

Hicks, E. P. & Robertson, R. C. (1927). The occurrence of paratyphoid C in Shanghai. *Chin. med. J.* **41**, 789.

Hobbs, B. C. (1964). Contamination of meat supplies. Paper read before the Health Congress, Roy. Soc. Hlth at Torquay, 27 April to 1 May 1964.

Hsu, H. C. & Chang, C. (1957). Clinical analysis of *Salmonella choleraesuis* infection. *Chin. J. intern. Med.* **5**, 614 (in Chinese).

Huang, C. H., Chang, H. C. & Lieu, V. T. (1937). Salmonella infection; a study of 17 cases of *Salmonella enteritidis* septicaemia. *Chin. med. J.* **52**, 345.

Huang, C. T. & Chan-Teoh, C. H. (1964). Salmonella serotypes isolated in Hong Kong. *J. trop. Med. Hyg.* **67**, 95.

Jen, F. P. & Pan, Y. T. (1955). *Salmonella suipestifer* infection: a report of 2 cases. *Chin. J. intern. Med.* **3**, 446 (in Chinese).

Kauffmann, F. (1954). *Enterobacteriaceae*, 2nd ed. Copenhagen: Ejnar Musksgaard Publisher.

Ku, C. C., Li, Y. S. & Cheng, J. H. (1955). *Salmonella suipestifer* infection in Wuhan: a clinical report. *Chin. J. intern. Med.* **3**, 918 (in Chinese).

Le Minor, L. (1964). 'Les Salmonelles en Indochine et en Chine. In *The World Problem of Salmonellosis*, pp. 530–8. Ed. E. Van Oye. The Hague: Dr W. Junk.

LIAO, T. C. (1957). Preliminary study of 113 strains of *Salmonella choleraesuis* in Kweiyang. *Nat. med. J. China* **43**, 431 (in Chinese).

LINDBERG, R. B. & BAYLISS, M. (1946). Salmonella types of the Pacific area. *J. infect. Dis.* **76**, 91.

LING, C. C., HSUEH, P. C., TAUER, S. S. & YANG, S. Y. (1941). Systemic salmonella infections with positive medullocultures. *Chin. med. J.* **59**, 45.

LIU, T. S. (1956). *Salmonella choleraesuis* infection in children. *Chin. J. Paed.* **7**, 277 (in Chinese).

LU, S. T. & YEH, T. T. (1955). Salmonella infection. *Chin. med. J.* **73**, 412.

NGUYEN-HUU-LUONG & FOURNIER, J. (1960). Abcès du foie et septicémie à *Salmonella cholerae-suis* chez un Montagnard du Lang Bian. La question des abcès bactériens du foie au Sud Viet-Nam. *Bull. Soc. Path. exot.* **53**, 256.

RAYNAL, J. H. & FOURNIER, J. (1947). Les salmonelles à Changhai (dans la pratique de l'Institute Pasteur de 1938 à 1945). *Méd. trop. Marseilles* **7**, 199.

SAKAZAKI, R. & NAKAYA, R. (1964). Epidemiological and ecological studies of salmonella in Japan. *Endem. Dis. Bull. Nagasaki Univ.* **6**, 167.

SAPHRA, I. & WASSERMANN, M. (1954). A clinical and epidemiological evaluation of 329 infections identified between 1940 and 1954 in the New York Salmonella Center. *Am. J. med. Sci.* **228**, 525.

SAPHRA, I. & WINTER, W. (1957). Clinical evaluation of 7,779 human infections identified at the New York Salmonella Center. *New Engl. J. Med.* **236**, 1128.

SMITH, H. WILLIAMS (1959). The isolation of salmonellae from the mesenteric lymph-nodes and faeces of pigs, cattle, sheep, dogs and cats and from other organs of poultry. *J. Hyg., Camb.* **57**, 266.

SMITH, H. WILLIAMS (1965). The immunization of mice, calves and pigs against *Salmonella dublin* and *S. cholerae-suis* infections. *J. Hyg., Camb.* **63**, 117.

TENBROECK, C., LI, C. P. & YÜ, H. (1931). Studies on paratyphoid C bacilli isolated in China. *J. exp. Med.* **53**, 307.

TSOU, C. (1959). Clinical observation of 30 cases of *Salmonella choleraesuis* infection in children in Wuhan. *Chin. J. Paed.* **10**, 241 (in Chinese).

VAN OYE, E. (edited by) (1964). *The World Problem of Salmonellosis.* The Hague: Dr W. Junk.

WANG, H. H., PENG, H. H., HSIEH, F. H. & TANG, T. H. (1957). Septicaemia of *Salmonella choleraesuis* complicated with pathological femur fracture: A case report. *Nat. med. J. China* **43**, 44 (in Chinese).

WILSON, G. S. & MILES, A. A. (1964). *Topley and Wilson's Principles of Bacteriology and Immunity*, 5th ed. p. 895. London: Edward Arnold.

WU, C. J. & ZIA, S. H. (1935). Systemic salmonella infections in man. *Chin. med. J.* **49**, 1217.

WU, W. Y. & FOURNIER, J. (1950). Notes on *Salmonella cholerae-suis kunzendorf* infection. Report of a case treated with Chloromycetin. *Chin. med. J.* **68**, 193.

ZIMMERMAN, L. E., COOPER, M. & GRABER, C. D. (1952). Bacteriologic studies in an outbreak of salmonellosis in Korea with special attention to *S. paratyphi* and perforations of paratyphoid ulcers. *Am. J. Hyg.* **56**, 252.

J. Hyg., Camb. (1967), **65**, 165
Printed in Great Britain

The sensitivity of the biotypes of *Brucella abortus* to three antibiotics used in selective media, and the description of a new biotype

BY I. D. FARRELL AND L. ROBERTSON
Public Health Laboratory, Preston

(*Received* 7 *December* 1966)

Brucella abortus can be isolated on selective agars from samples of untreated cows' milk and from other contaminated sources. The selective agars in current use are modifications of the one described by Kuzdas & Morse (1953). This medium contains a combination of three antibacterial antibiotics—polymyxin B sulphate, circulin, and bacitracin; an anti-fungal antibiotic—actidione; and an aniline dye—ethyl violet, to inhibit the growth of contaminant organisms.

If the composition of the selective agars used for the isolation of *B. abortus* from 1953 onwards is studied, it will be noted that two recent additions to the collection of antibacterial and anti-fungal antibiotics incorporated in the agars are nitrofurfuryl methyl ether (Morris, 1956) and amphotericin B (Report, 1964*a*). Otherwise it appears that each successive medium has been elaborated from that described by Kuzdas & Morse and neither in the original nor in any subsequent publications has experimental evidence been given to show that the concentrations of antibiotics used were in fact the most effective for the purpose. Therefore it was decided to investigate the two selective agars used in this laboratory, viz. Mair's medium (1955) and a modification of Morris's medium (1956), in order to determine whether they contained the optimal concentrations of antibiotics necessary for the isolation of *B. abortus* from contaminated material.

During the course of the investigation, which is not yet complete, it has become apparent that there were certain similarities between the antibiotic-sensitive strains and the dye-sensitive strains of *B. abortus*.

The object of this communication is to report the sensitivity of biotypes of *B. abortus* to the three antibiotics, polymyxin B sulphate, bacitracin, and amphotericin B; and to propose that the dye-sensitive variant of biotype 4 should be considered as a new biotype.

MATERIAL AND METHODS

The following 219 strains of *Brucella* were examined during the course of the investigation:—

(*a*) Ten strains of the prototypes belonging to the WHO collection, viz. *B. abortus*, biotypes 1, 2, 3, 4, 5, 6, 7, 9; (biotype 8 was not available); *B. melitensis*, biotype 1, and *B. suis*, biotype 1.

(*b*) Two hundred and nine 'wild' strains isolated from cattle in Lancashire.

All strains were maintained on serum-dextrose agar slopes and kept at a tempera-

ture of 4° C. When required for investigations they were subcultured on 5 % blood agar and incubated in air containing 20 % carbon dioxide for 3 days at 37° C.

Typing

The biotype of each strain of *Brucella abortus* was determined on the basis of the following criteria (Wilson, 1933; Cruickshank, 1954; Report, 1964*b*):

(1) Carbon dioxide dependence of the primary isolate.

(2) Production of hydrogen sulphide.

(3) The sensitivity of the organism to the two aniline dyes—basic fuchsin and thionin at the concentrations recommended by the Expert Committee on Brucellosis.

(4) The ability of a suspension of the organism to agglutinate with *Brucella abortus* and *Brucella melitensis* monospecific sera.

(5) The sensitivity of *Brucella abortus* bacteriophage.

Antibiotics

Polymyxin B sulphate was supplied in glass vials containing 500,000 international units (i.u.) as a dry sterile powder (Burroughs Wellcome). A stock solution containing 10,000 i.u./ml. was prepared in sterile distilled water.

Amphotericin B (Fungizone) was supplied in glass vials containing 50 mg. of sterile dry powder (E. R. Squibb and Sons). A stock solution of 5000 μg./ml. was prepared in sterile distilled water.

Bacitracin was supplied as an unsterile dry powder (Calmic Ltd.). A stock solution of 8,000 i.u./ml. was prepared and sterilized by Seitz filtration.

Fresh solutions of antibiotics were prepared for each batch of ditch plates.

Technique for determining the antibiotic sensitivity of the various strains

An agar diffusion (ditch plate) technique was employed to determine the sensitivity of each strain of *B. abortus* to the antibiotics polymyxin B sulphate, amphotericin B, and bacitracin.

All glassware was washed and sterilized by autoclaving at 15 pounds per square inch for 15 min. Plastic Petri dishes sterilized by gamma-radiation were used throughout the experiments; the bottoms of these dishes have a flat surface thus giving an agar plate of uniform depth.

Serum-dextrose agar was poured into 70 mm. diameter Petri dishes to a depth of about 6 mm. A strip of agar, 15 mm. wide, was removed from each plate. The central ditch thus formed was filled with serum-dextrose agar containing an appropriate amount of antibiotic solution. The range of concentrations of antibiotics against which each strain was titrated was selected so as to give a range of doubling dilutions which started at the concentration of the particular antibiotic used in the selective agars. In the case of polymyxin B sulphate seven dilutions were used, ranging from 5 to 320 i.u./ml.; for amphotericin B six dilutions were used, ranging from 10 to 320 μg/ml.; for bacitracin seven dilutions were used, ranging from 25 to 1600 i.u./ml

Diffusion of the antibiotic from the central ditch was allowed to take place

overnight at a temperature of 4° C., after which time an equilibrium state was assumed to have been reached (Ericsson, 1960). Using smooth colonies, a suspension of the organism was prepared in sterile physiological saline to give a density comparable with a Brown's opacity tube no. 2. A standard loopful of a suspension of each strain was streaked across the ditch plate at right-angles. Eight strains were tested on each ditch plate.

The performance of each plate was controlled by inoculating suspensions of the WHO prototype strains of *B. abortus*, biotype 1, and *B. abortus*, biotype 2, prepared in the same way, across each plate.

Bacitracin and amphotericin B diffuse into the medium and in higher concentrations inhibit growth some distance from the ditch. Polymyxin B sulphate, on the other hand, diffuses hardly at all even though time to reach an equilibrium state has been allowed and the only possible endpoint reading for this antibiotic is inhibition of growth over the ditch. So that the endpoint readings for the three antibiotics would be uniform, the endpoint of each titration was expressed as the lowest concentration which completely inhibited growth of the organism over the area of the ditch itself.

Each suspension was also inoculated on serum-dextrose agar to check its purity and viability. The plates were incubated in air containing 20 % carbon dioxide at a temperature of 37° C. for 3 days.

RESULTS

Table 1 shows the endpoint titrations for the eight WHO biotypes of *B. abortus*, *B. melitensis*, biotype 1, and *B. suis*, biotype 1, to the antibiotics polymyxin B sulphate, amphotericin B, and bacitracin. This shows that the WHO prototype of *B. abortus*, biotype 2, differs from all the other biotypes and from *B. melitensis* and *B. suis* in being much more sensitive to the three antibiotics polymyxin B sulphate, amphotericin B and bacitracin.

Table 1. *The sensitivity of WHO prototype strains to polymyxin B sulphate, amphotericin B and bacitracin*

Organism	Biotype	Polymyxin (i.u./ml.)	Amphotericin (μg./ml.)	Bacitracin (i.u./ml.)
Brucella abortus	1	160	$\geq$ 320	1600
	2	80	40	100
	3	320	$\geq$ 320	800
	4	320	$\geq$ 320	800
	5	320	$\geq$ 320	800
	6	320	$\geq$ 320	800
	7	320	$\geq$ 320	800
	9	160	$\geq$ 320	800
B. melitensis	1	$\geq$ 320	$\geq$ 320	800
B. suis	1	$\geq$ 320	$\geq$ 320	800

Table 2 is a synoptic presentation of the endpoint titrations obtained for 209 'wild' strains of the biotypes isolated from cattle in the north of Lancashire.

With the exception of strains of biotype 2 and the dye-sensitive variant of biotype 4 the strains of other biotypes were sensitive to 160 or 320 i.u./ml. poly-

Table 2. *The sensitivity of 209 wild strains of* Brucella abortus *to polymyxin B sulphate, amphotericin B, and bacitracin (number of strains sensitive to the different concentrations)*

Biotype	Polymyxin (i.u./ml.)							Amphotericin B (μg./ml.)							Bacitracin (i.u./ml.)						
	5	10	20	40	80	160	320	10	20	40	80	160	320	> 320	25	50	100	200	400	800	1600
Brucella abortus biotype 1, 24 strains	0	0	0	0	0	13	11	0	0	0	0	0	0	24	0	0	0	0	0	12	12
B. abortus biotype 2, 126 strains	0	0	0	2	100	24	0	1	3	92	19	1	0	10	1	6	89	28	2	0	0
B. abortus biotype 4, 11 strains	0	0	0	0	0	6	5	0	0	0	0	0	0	11	0	0	0	0	1	5	5
B. abortus, biotype dye sens. 4, 8 strains	0	0	0	0	2	6	0	0	0	8	0	0	0	0	0	0	2	6	0	0	0
B. abortus, biotype 5, 20 strains	0	0	0	0	0	19	1	0	0	0	0	0	0	20	0	0	0	0	0	20	0
B. abortus, biotype 9, 20 strains	0	0	0	0	0	20	0	0	0	0	0	0	0	20	0	0	0	0	0	20	0

320 μg./ml. is the limit of the solubility of amphotericin B in the medium.

Table 3. *The biochemical and serological classification of the biotypes of* Brucella abortus *isolated from cattle in Lancashire and including* Brucella melitensis, *biotype* 1 *and* Brucella suis, *biotype* 1, *for comparison*

Species	Biotype	Phage Tb at R.T.D.	CO_2 requirement	H_2S production	Growth on dyes		Agglutination with monospecific sera		Remarks
					Thionin	Basic fuchsin	Abortus	: Melitensis	
Brucella abortus	1	+	+	+	−	+	+	−	Typical abortus
	2	+	+	+	−	−	+	−	Dye sensitive
	4 (*a*)	+	+	+	−	+	−	+	Abortus biochem. and melitensis serological
	4 (*b*)	+	+	+	−	−	−	+	As 4 (*a*) but dye sensitive
	5	+	−	−	+	+	−	+	British melitensis
	9	+	−	+	+	+	−	+	Similar to British melitensis but produces H_2S
B. melitensis	1	−	−	−	+	+	−	+	.
B. suis	1	−	−	+	+	−	+	−	.

myxin B sulphate, were resistant to 320 μg./ml. of amphotericin B, and all but one of the strains were sensitive to 800 or 1600 i.u./ml. of bacitracin. The greater antibiotic sensitivity of biotype 2 is also revealed in Table 2. Out of 126 strains of biotype 2, 102 were more sensitive to polymyxin B sulphate, 116 were more sensitive to amphotericin B, and all of the 126 strains were more sensitive to bacitracin than strains of the other biotypes with one exception, viz. a variant of *B. abortus*, biotype 4, which is also dye sensitive (Table 3), which was found to be a little more sensitive to polymyxin B sulphate and much more sensitive to amphotericin B and bacitracin.

DISCUSSION

The genus *Brucella* is divided into three species: *B. abortus*, *B. melitensis* and *B. suis*. The system of classification depends upon the results of oxidative metabolic tests with a series of amino acids and the sensitivity of the organisms to the *B. abortus* bacteriophage.

There are nine biotypes of *B. abortus* and their classification is dependent upon the quantitative rather than the qualitative interpretation of the results of the following tests: CO_2 dependence of the primary isolate; production of H_2S; sensitivity to the two aniline dyes, basic fuchsin and thionin; agglutination reaction with monospecific abortus sera and monospecific melitensis sera (Report, 1964*b*).

The WHO method of classification of the biotypes present in the cattle population of the north of Lancashire is shown in Table 3, which also includes *B. melitensis* biotype 1, and *B*, *suis*, biotype 1, for comparison.

It is apparent from Table 3 that the dye-sensitive strains belong to biotype 2 and include the dye-sensitive variants of biotype 4, and that they are identified from one another by their reaction with monospecific abortus and monospecific melitensis sera.

It is evident from the results shown in Table 2 that biotypes of *B. abortus* can be divided into two classes, those that are sensitive to bacitracin and amphotericin B and those which are comparatively resistant to bacitracin and amphotericin B.

The majority of strains of biotype 2 and the dye-sensitive variants of biotype 4 were particularly sensitive to amphotericin B and bacitracin. All but two strains (132/134) were sensitive to a concentration of 200 i.u./ml. of bacitracin, and 123/134 strains were sensitive to 80 μg./ml. of amphotericin B. However, strains of the remaining biotypes were noticeably more resistant to both of these antibiotics (Table 1 and Table 2).

Ten strains of biotype 2 gave an antibiotic sensitivity pattern not seen in the other 116 strains of biotype 2—they were sensitive to concentrations of 200 i.u./ml. of bacitracin but were resistant to amphotericin B in concentrations as high as 320 μg./ml. These results would seem to form the basis of a subdivision of biotype 2, which could be of epidemiological importance, and, in fact, even when there are minor variations in antibiotic sensitivity pattern in strains of biotype 2 from different cows, we have always found that successive strains from any one individual cow have given a constant sensitivity pattern.

The amphotericin B resistant strains of biotype 2 may be mutants selected

through laboratory adaptation, but this seems unlikely as amphotericin B is not employed in any of the media used in this laboratory for the isolation of *B. abortus*, and also it is most unlikely that the animals would have come into contact with this antibiotic in nature. It may be that these variants of biotype 2 have arisen purely from natural mutation of the genes controlling the characteristics of the organism. It is of interest at this point to mention that Cruickshank (1954) described the subdivision of biotype 2 on the basis of pyronin sensitivity. It is hoped to obtain a larger collection of these variants and to compare their antibiotic and dye sensitivities.

The WHO prototype of biotype 4 and the 'wild' strains tested were sensitive to concentrations of 1600 i.u./ml. of bacitracin and were resistant to concentrations of amphotericin B as high as 320 μg./ml. On the other hand, the dye-sensitive variants of biotype 4 were sensitive to concentrations of 200 i.u./ml. of bacitracin and 40 μg./ml. of amphotericin B; they are as sensitive to these antibiotics as most strains of biotype 2. These dye-sensitive variants of biotype 4 are unusual in that their antibiotic sensitivity is completely different from that of the WHO prototype of biotype 4.

In this laboratory selection of resistant mutants from agars containing the two aniline dyes, basic fuchsin and thionin, and investigation of any change in the antibiotic sensitivity of the mutant, has shown that the dye sensitivity of a brucella strain can be altered without changing its antibiotic sensitivity. These results would seem to indicate that antibiotic sensitivity and the dye sensitivity of brucella strains are not genetically related properties.

The only difference between biotype 1 and biotype 2 is that biotype 2 is dye-sensitive, and this one property has been sufficient for them to be recognized as two distinct biotypes.

The dye-sensitive variants of biotype 4 are different from the WHO prototype of biotype 4 in both their antibiotic sensitivity and dye sensitivity—two genetically unrelated characteristics. In the light of this evidence it is suggested that dye-sensitive variants of biotype 4 should be reclassified. These variants seem more closely related to biotype 2 than to biotype 4; but as the classification of *B. abortus* into its nine biotypes is more a matter of degree than of positive and negative characteristics it is further suggested that this variant be raised to the status of a biotype and should be called biotype 10.

SUMMARY

An investigation into the antibiotic sensitivity of *B. abortus* biotypes to polymyxin B sulphate, amphotericin B, and bacitracin has shown that the dye-sensitive strains are also antibiotic-sensitive; and preliminary experiments suggest that these two properties are not genetically related.

The results suggest that biotype 2 can be subdivided on the basis of antibiotic sensitivity pattern, and that this could be of epidemiological significance.

Investigation into the antibiotic sensitivity pattern of the dye-sensitive variant *B. abortus*, biotype 4, leads us to believe that this strain should be reclassified as a separate biotype and provisionally called biotype 10.

We wish to thank the Medical Officers of Health and their Public Health Inspectors for their co-operation in obtaining the samples of milk from which the Brucella organisms were isolated. We should like to record our appreciation of the technical assistance of Mr F. D. Hargreaves.

REFERENCES

CRUICKSHANK, J. C. (1954). Observations on brucella species based on the examination of 800 strains. *J. Hyg., Camb.* **52**, 105.

ERICSSON, H. (1960). Laboratory Methods: determination of bacterial sensitivity. *Scand. J. clin. Lab. Invest.* (*Suppl.*) **60**, 9.

KUZDAS, C. D. & MORSE, E. V. (1953). A selective medium for the isolation of brucellae from contaminated materials. *J. Bact.* **66**, 502.

MAIR, N. S. (1955). A selective medium for the isolation of *Brucella abortus* from herd samples of milk. *Mon. Bull. Minist. Hlth* **14**, 184.

MORRIS, E. J. (1956). A selective medium for *Brucella* spp. *J. gen. Microbiol.* **15**, 629.

REPORT (1964*a*). Brucellosis in the British Dairy Herd. *Animal Disease Survey* no. 4, H.M.S.O.

REPORT (1964*b*). Joint FAO/WHO Expert Committee on Brucellosis. *Tech. Rep. Ser. Wld Hlth Org.* no. 289, Annex 2, p. 50.

WILSON, G. S. (1933). The classification of the brucella group: a systematic study. *J. Hyg., Camb.* **33**, 516.

J. Hyg., Camb. (1967), **65**, 173
Printed in Great Britain

The progression of herpes simplex virus to the central nervous system of the mouse

BY PETER WILDY

Department of Virology and Bacteriology, The Medical School, Birmingham 15

(*Received* 15 *December* 1966)

The mechanisms by which a virus may penetrate from the periphery of an animal to the central nervous system have interested pathologists for many years. There exists a voluminous literature upon the subject which has been comprehensively reviewed from time to time (Craigie, 1937; Sabin, 1939; Faber, 1953; Rhodes, 1953; Wright, 1953; Burnet, 1955). Although most of the work concerned poliomyelitis virus, the spread of herpes simplex virus to the central nervous system of the rabbit was also extensively studied.

As a result of studies with herpes virus in rabbits two rival hypotheses were formulated; these profoundly influenced our concepts of how other viruses and even toxins might reach the central nervous system. In the first (Goodpasture & Teague, 1923), virus was supposed to ascend from the periphery by way of the axon cylinder and in the second (Marinesco & Dragonesco, 1923), by way of the tissue spaces between the axons. Both hypotheses satisfactorily explained the early localization of lesions to those segments of the central nervous system anatomically connected with the site of inoculation.

A third hypothesis, originally suggested by Doerr & Vochting (1920) and later reconsidered by Field (1953), was that virus invaded the central nervous system from the blood-stream. Following the demonstration that viraemia occurred in both experimental and in naturally acquired poliomyelitis (Horstmann, McCollum & Mascola, 1954; Melnick, 1945; Ward, Horstmann & Melnick, 1946; Wenner & Kamitsuka, 1957) this third hypothesis became important. But although it explained how virus could reach the central nervous system, it did not explain the localization of tissue damage to a few segments of the cerebrospinal axis. A further difficulty was that paralysis in poliomyelitis was not commonly associated with those segments adjacent to the *area postrema* or to the other weak points in the haematoencephalic barrier (cf. Bodian, 1952). But when it was recognized that localized paralysis in poliomyelitis was sometimes precipitated by injections of diphtheria prophylactics and the like (Martin, 1950; McCloskey, 1950) it was suggested that a reflex mechanism might in some way predispose to a local invasion of the nervous system from the blood-stream (Burnet, 1950). That such localized reflexes occurred was shown by Field, Grayson & Rogers (1949, 1951).

Despite the large volume of published work on the means by which viruses reach the central nervous system from the periphery, little of the earlier work appears satisfactory. First, few studies were made using enough experimental animals to give confidence in the conclusions drawn. Secondly, too much reliance was often

placed on results obtained using only one criterion for assessment (e.g. development of paralysis, the presence or absence of infective virus or the presence or absence of histological change) and, thirdly, in many instances excessive preoccupation with one particular process prevented enough attention being paid to others. These criticisms do not apply to the more recent studies (e.g. Wenner & Kamitsuka, 1957).

It was originally intended to make a full study of events occurring in an experimental system in which herpes simplex virus passed from the periphery to the central nervous system of the mouse, using large numbers of animals and several different quantitative criteria for assessment. It was not possible to carry this out fully and the work was shelved. But recently the interesting studies of Johnson (1964*a*, *b*), Platt (1964) and Yamamoto, Otani & Shiraki (1965) have prompted the belief that these incomplete findings may now be of some value.

MATERIALS AND METHODS

Virus

Strains

The principal strain of virus used was HFEM whose characteristics have been described elsewhere (Wildy, 1955). It was maintained by intracerebral passage in mice. The strain Nag was originally isolated at the Walter and Eliza Hall Institute, Melbourne, from a case of herpes labialis and was maintained by passage on the chorioallantois.

Suspending fluid

All operations in which it was desired to preserve the infectivity of virus were carried out at 0° C. using broth containing penicillin (12·5 units/ml.) and streptomycin (5 μg./ml.).

Egg techniques

Conventional egg techniques were used (Beveridge & Burnet, 1946) and require no further description.

Maintenance and preparation of inocula

Stock suspensions of strains were kept in ampoules at $-70°$ C. Inoculating suspensions were made from these by Nagler's (1946) method in which eggs at the 8th day of incubation were inoculated by way of the yolk sac with 10^{-2} dilution of stock virus. After incubation at 35° C. for 3 days, the amniotic fluid was collected, clarified by centrifugation and either used immediately or stored at $-70°$ C. until required.

Recovery of virus

Virus was extracted by grinding tissues with alundum. The procedure was standardized by grinding with a standard quantity of alundum, which had been damped with broth, making one hundred circular movements. One ml. broth was then added and the whole ground a further 20–30 times. The resulting suspension

was centrifuged at 2500 r.p.m. for 10 min. and usually titrated immediately, but occasionally the supernatant fluid had to be stored in ampoules at -70° C. before it was titrated.

Titration of infective virus

Infectivity titrations were made on the chorioallantois using the method of Burnet & Lush (1939*a*) and were expressed in terms of the pock unit (pock u.) which causes one lesion on the chorioallantois. For most purposes, tenfold dilutions were used and each inoculated on to four eggs, but in some experiments, where large numbers of titrations had to be made, it became impracticable to use more than two eggs per dilution. In such cases, samples of material were always set aside in ampoules and stored at -70° C. Where the eggs produced dissimilar results, the titration was repeated.

Sera

Specific neutralizing antisera were prepared in young adult rabbits as previously described (Wildy, 1954). Three graded weekly doses were given intradermally; (*a*) 2×10^5 pock u. virus inactivated with 0·01 % formalin; then (*b*) 2×10^2 pock u.; and (*c*) 2×10^5 pock. u infective virus. By the 4th week, all rabbits possessed strongly active neutralizing antisera as measured by the method of Burnet & Lush (1939*a*).

Mice

Strains

Most of the work done was in Australia using the Hall Institute strain of mice. For the experiments made in England, a strain which had originated from the Glaxo stocks (strong albino/2/G) was used. Both strains were used during the 5th week of life and both behaved similarly towards herpes simplex virus.

Injection and inoculation procedures

Conventional methods were used for inoculation by the intracerebral, intraperitoneal, intravenous, intranasal, intratesticular and corneal routes. Intraspinal inoculation was made by the method of Habel & Li (1951). To avoid artificial tensions in the tissues, intradermal inoculation was made by multiple pressure in a standard way. A drop of undiluted virus suspension was placed on the sole of one hind foot of the anaesthetized mouse and a blunt needle was pressed firmly through the drop fifty times. The sole was then rubbed firmly with the shank of the needle five or six times. If the skin was visibly broken, the mouse was discarded.

Taking tissue samples from mice

The method of removing pieces of tissue was mostly straightforward. However, the method used for removing samples from the central nervous system requires mention. After killing the mouse, it was pinned out in the prone position, and the whole of the dorsal skin was then removed. The skull cap was removed aseptically and the lumbar vertebrae divided. A syringe fitted with a wide bore needle was charged with saline and the needle inserted into the spinal canal. Moderate pressure

on the plunger forced the whole spinal cord through the foramen magnum. The entire central nervous system was then removed and divided into three portions. The cord was divided midway between the cervical and lumbar enlargements and the medulla oblongata was removed from the base of the brain. These portions will be referred to as the lumbar cord, cervical cord and brain. The sciatic nerve was then removed after splitting the gluteal muscles. The method is easy and quick, enabling large numbers of mice to be handled, but its main disadvantage is that the dorsal root ganglia are left behind in the carcass.

RESULTS

Virulence of virus inoculated intracerebrally

The first experiments were designed to evaluate the virulence of the virus to be used and to discover a method of peripheral inoculation which regularly leads to paralysis. The strain HFEM was already known to be highly virulent for mice when inoculated intracerebrally; one LD 50 $\approx$ 1 pock u. (Wildy, 1955). This conclusion was strengthened by determining the number of pock u. which constitute one LD 50 and comparing this value with the number required to infect 50% of mice (one ID 50). Tenfold dilutions of virus were each inoculated on the chorio-allantois of four eggs and into the brains of four mice in 0·05 ml. volumes. Pocks were counted after 48 hr. incubation at 35° C. The mice were examined daily for 14 days and the number of days elapsing between inoculation and death from encephalitis noted. On the 15th day, all surviving mice were challenged with 100 pock u. strain HFEM, which is sufficient to kill the normal mouse. These were then examined daily for a further period of 14 days. Mice which survived this challenge were regarded as having been immunized by the first inoculation, and those that did not were regarded as not having been infected by it. The results obtained with strains HFEM and Nag using Hall Institute mice and Glaxo mice show three points (Table 1). First, with both viral strains the ID 50 is close to one pock unit. Secondly, whilst strain Nag killed no mice even when 116 pock u. were inoculated, the LD 50 for strain HFEM was close to the ID 50 and thirdly, both mouse strains were about equally susceptible to strain HFEM when inoculated intracerebrally.

Virulence of HFEM virus inoculated intraspinally

It was of particular interest for us to know the virulence of virus when inoculated intraspinally, rather than intracerebrally, because it was the lumbar cord which was first invaded by virus in most experiments to be described. Unfortunately, the volumes of virus inoculated intraspinally, though of standard size, were so small that they were difficult to measure. For this reason virulence of virus inoculated in this way was not compared with egg titrations; falling dilutions of virus were inoculated intraspinally into groups of mice and the infectivity (ID 50, based as before on the results of inoculation and subsequent challenge) was compared with the ability to produce paralysis (PD 50) and with the LD 50. In two experiments carried out with different seeds of strain HFEM, it was found that the ID 50 and the PD 50 lay close together but that about ten times as much virus was required to constitute the LD 50 when inoculated intraspinally (Table 2).

Table 1. *Virulence for mice of virus inoculated intracerebrally*

Virus strain	Dilution	Chorioallantoic inoculation eggs (mean no. pocks)	Mouse strain	Intracerebral inoculation mice
HFEM	10^{-5}	0·0	Hall Institute	I, S, S, S
	10^{-4}	0·25		7, 8, S, S
	10^{-3}	2·14		7, 9, 10, I
	10^{-2}	26·5		4, 5, 6, 7
Nag	10^{-5}	0·0	Hall Institute	S, S, S, S
	10^{-4}	0·25		I, I, S, S
	10^{-3}	18·0		I, I, I, S
	10^{-2}	116·0		I, I, I, I
HFEM	10^{-7}	0·0	Glaxo	I, S, S, S
	10^{-6}	0·75		6, 7, 10, 13
	10^{-5}	7·3		6, 6, 8, 11

Results of inoculating dilutions of virus in parallel on the chorioallantoic membrane and intracerebrally into mice. Last column figures, day of death after incoculation; S, mouse survived 14 days but susceptible to challenge with 100 pock u. HFEM; I, mouse survived 14 days and immune to challenge.

Table 2. *Virulence for mice of strain HFEM inoculated intraspinally*

	Dilution	Day of paralysis	Day of death and Result of challenge
Expt. 1	10^{-7}	6, n.p., n.p., n.p.	I, S, S, S
	10^{-6}	7, 7, n.p., n.p.	I, I, I, I
	10^{-5}	3, 4, 4, 4	5, 6, I, I
Expt. 2	10^{-5}	n.p., n.p., n.p., n.p.	S, S, S, S
	10^{-4}	3, 8, 9, 10, n.p.	6, 10, I, I, I
	10^{-3}	3, 3, 8, n.p., n.p.	6, 6, I, I, I
	10^{-2}	2, 3, 3, 5	6, 5, 5, 6

Results of inoculating tenfold dilutions of two different seeds of HFEM intraspinally. Middle column figures, day after inoculation upon which paralysis was noted; n.p., not paralysed within 14 days. Third column refers to the same mice: symbols as in Table 1.

Table 3. *Virulence of virus inoculated peripherally*

Route of inoculation	Method	Inoculum size	Proportion of mice developing paralysis	Proportion of mice which died
Intravenous	Syringe	$2{\cdot}3 \times 10^6$ pock u.	0/4	2/4
Intraperitoneal	Syringe	$2{\cdot}3 \times 10^8$ pock u.	1/4	1/4
Intratesticular	Syringe	$2{\cdot}3 \times 10^6$ pock u.	0/4	0/4
Corneal	Scarification	$4{\cdot}6 \times 10^7$ pock u./ml.	0/4	0/4
Intradermal	Multiple pressure	$4{\cdot}6 \times 10^7$ pock u./ml.	1/10	1/10

Results of inoculating strain HFEM into mice in various ways.

Virulence of HFEM virus inoculated peripherally

Plainly, strain HFEM was highly virulent when inoculated into the central nervous system; this was not the case when it was inoculated peripherally (Table 3). In several experiments large doses of virus were inoculated by various routes. No effects followed inoculation on the cornea or into the testicle; when large amounts of virus were inoculated intravenously, paralysis did not occur but two of the four mice died (the post-mortem findings are mentioned later). One of four mice inoculated intraperitoneally with a massive dose of virus and only one of ten mice inoculated intradermally became paralysed and died. The one mouse which became paralysed is of interest because it showed that HFEM might be used to investigate the path of viruses to the central nervous system of mice. The mouse, which had been inoculated on the sole of the right forefoot, developed paralysis in the right arm 6 days later. This rapidly progressed and on the following day the animal died. The infected forefoot and portions of the central nervous system were titrated for infective virus with the following result: forefoot, 7400 pock u.; lumbar cord, 240 pock u.; cervical cord, 1280 pock u.; cerebellum, 1200 pock u.; cerebral hemispheres, 100 pock u.

Table 4. *Modification of the results of intradermal inoculation by injecting 0·1 ml. 10 % sodium chloride 24 hr. beforehand*

Inoculum concentration (pock u./ml.)	Mice pretreated with 10 % sodium chloride		Control mice	
	Vesicle formation	Onset of paralysis	Vesicle formation	Onset of paralysis
$4{\cdot}6 \times 10^3$	6, 7, –, –	9, 11, –, –	–, –, –, –	–, –, –, –
$4{\cdot}6 \times 10^5$	6, –, –, –	11, –, –, –	–, –, –, –	–, –, –, –
$4{\cdot}6 \times 10^7$	3, 3, 3, 6	7, 7, 7, 7	7, –, –, –	–, –, –, –
$4{\cdot}6 \times 10^9$	3, 3, 3, 3	7, 7, 7, 7	3, 3, 3, –	7, 8, –, –

Figures indicate day after inoculation upon which vesicle formation or paralysis was first noted. – indicates negative findings up until 14 days.

Virulence of HFEM virus for mice previously treated with hypertonic saline

Olitsky & Schlesinger (1941) found that the incidence of paralysis was greater when herpes simplex virus was inoculated into an area of skin which had previously been injected subcutaneously with a small quantity of 10 % saline. This effect was well shown when four dilutions of virus were each inoculated by multiple pressure into the soles of four normal mice and of four mice which had 24 hr. previously been given a subcutaneous injection of 0·1 ml. 10 % saline (Table 4). The amount of virus required to give local vesicular lesions and paralysis after such pretreatment was about one-hundredth of that required for the control mice.

It appeared, therefore, that this technique (the *standard procedure*) of inoculating concentrated virus by multiple pressure into the skin of the sole of a hind foot previously treated with hypertonic saline provided a satisfactory system for studying the route by which a virus invades the central nervous system from the periphery.

The histological changes due to infection of the central nervous system with strain HFEM

It had been expected that the central nervous system of mice ill with herpetic encephalomyelitis would show well marked histological evidence of infection, but disappointingly only slight histological changes were found. Ten mice were infected intracerebrally with doses of virus ranging from 2×10^2 to 2×10^6 pock u. strain HFEM and each was killed when showing advanced clinical encephalitis 2–8 days later. The brains were removed immediately and fixed whole in 10% formol saline. Sections were cut at various levels, stained with haematoxylin and eosin, and thoroughly examined. Varying degrees of leptomeningitis and small intracerebral haemorrhages at the site of incoculation were noted. In all mice chromatolysis and nuclear ballooning was observed in some nerve cells but no inclusion bodies were seen, and there were no perivascular cellular infiltrations. Because of the slight and indefinite nature of the changes, it was not possible to correlate intensity of reaction with dose of virus nor with the interval since inoculation.

The spinal cords of 20 mice were similarly examined between 2 and 6 days after peripheral inoculation by the standard procedure. The histological picture was again indefinite. Similar nuclear changes were observed in some anterior horn cells and after 4 or 5 days in some glial cells. Two mice which had been inoculated by the standard procedure were killed 4 and 6 days later, and after decalcification were sectioned in such a way as to cut through the posterior root ganglia. No histological abnormality whatever was found. For these reasons histological methods have not been used.

Distribution of infective virus after peripheral inoculation

Twenty-four mice were inoculated by the standard procedure in the sole of the left foot with a suspension of strain HFEM containing $2 \cdot 3 \times 10^8$ pock u./ml. At daily intervals for 5 days four mice were taken at random and killed. Infective virus was titrated in the foot, sciatic nerve, lumbar cord, cervical cord and brain. The remaining group of four mice was observed for 14 days and in all cases paralysis ensued, appearing first in the inoculated limb at the 6th (2), 7th and 9th days after infection. The total amount of infective virus in the foot had reached a high value 24 hr. after infection (Table 5). There was a slight increase in the next 24 hr. which was followed by slow decline in the total amount of infective virus present. Virus was found in the central nervous systems of three of four mice on the second day, and as expected, was first demonstrated in the lumbar cord. On succeeding days it was found in the cervical cord and brain, suggesting that it had progressed up the cerebro-spinal axis. Viraemia was demonstrated in two of four mice on the 2nd day, and thereafter low levels were regularly found.

These results suggested that virus had entered the central nervous system by way of the sciatic nerve, which was found to contain infective virus from the second day. But it was possible also that virus had passed via the blood to the lumbar cord by a mechanism such as was suggested by Burnet (1950), or by other routes such as by the lymphatic vessels or even by simple extension through other tissues

which were not examined in this experiment. Accordingly the possible roles of the blood, the lymphatic system, other intervening tissues and the peripheral nerve were investigated as potential transporters of virus.

Table 5. *Distribution of infective virus in various situations after inoculation of virus intradermally into the sole of the right hind foot which had been pretreated with* 10 % *sodium chloride*

Day	Right foot	Right sciatic nerve	Lumbar cord	Cervical cord	Brain	Blood
1	5·20	0·95	< 0	< 0	< 0	< 0
	5·65	< 0	< 0	< 0	< 0	< 0
	5·70	< 0	< 0	< 0	< 0	< 0
	4·00	< 0	1·08	< 0	< 0	< 0
2	4·00	1·62	0·00	< 0	< 0	1·15
	6·36	—	0·73	< 0	< 0	< 0
	6·15	2·19	0·70	< 0	< 0	< 0
	6·36	2·00	< 0	< 0	< 0	0·90
3	4·78	3·30	3·00	2·00	1·04	0·48
	3·60	4·90	4·56	1·04	0·90	0·48
	6·23	5·70	2·55	1·67	0·85	0·30
	0·95	5·85	3·90	1·95	0·70	0·85
4	5·95	4·59	3·60	1·23	1·00	0·90
	4·94	4·51	—	0·70	< 0	0·60
	4·36	6·00	7·60	5·30	1·21	0·70
	4·38	1·96	1·23	0·00	< 0	0·70
5	6·08	2·25	3·30	1·65	0·95	0·70
	1·30	1·10	0·90	1·63	0·00	—
	4·82	3·00	2·30	1·98	< 0	0·48
	< 0	1·85	1·20	0·90	< 0	—

Results of inoculating a suspension of strain HFEM containing $2·3 \times 10^8$ pock u./ml. by multiple pressure. Values = $\log_{10}$ pock u./0·1 ml. organ extract; –, not done.

Role of the blood

Two series of experiments were done to determine the role of the blood as a vehicle for the carriage of virus to the central nervous system; first, the effects of an artificial viraemia were observed and secondly, an attempt was made to evaluate the importance of the viraemia that occurred after peripheral inoculation in the subsequent chain of events.

Effects of artificial viraemia

Viraemia regularly occurred after standard peripheral inoculation (Table 5), yet virus inoculated intravenously killed mice without producing paralysis (Table 3). The next points examined were the rate at which virus disappeared from the circulation and the fate of virus that had been inoculated intravenously.

Rate of disappearance of virus from the circulation after intravenous inoculation. A known amount of virus was injected into the tail veins of pairs of mice and at intervals the mice were bled. The blood was defibrinated by whipping, a procedure which had been shown not to alter the expected infective titre when virus and freshly drawn blood were mixed. The samples were at once titrated for infective

virus. The geometric mean of values was obtained for each pair of samples and from this, the total circulating virus was calculated on the assumption that the blood volume was 2·0 ml. Virus was removed very rapidly from the bloodstream in the first few minutes (Fig. 1); thereafter steadily decreasing amounts of infective virus were found up to the fourth hour and subsequently no virus was detected up to the 8th hr. of the experiment.

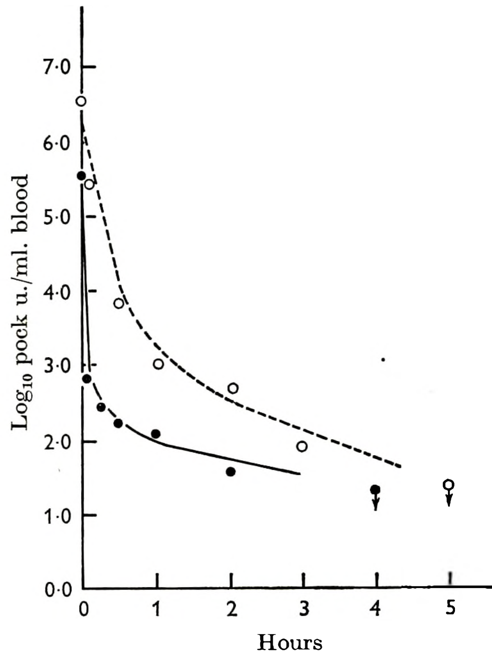


Fig. 1. The rate of disappearance of virus from the circulation of the mouse after intravenous inoculation of suspension of HFEM. Two experiments are shown.

Fate of intravenously inoculated virus. The common macroscopic appearances after intravenous inoculation of virus were varying degrees of congestion of the lung and of fatty change in the liver. Haematoxylin and eosin stained sections of lung showed gross congestion with haemorrhage into the alveoli; there was no evident increase in the number of inflammatory cells and no cytopathic changes were seen. The bronchi appeared normal with some serous secretion in their lumina. Sections of liver showed some evidence of fatty change and perivascular cells, lymphocytes, fibroblasts and small round cells. Sections of kidney, spleen and pancreas showed no abnormality.

One striking feature of these experiments was that unless massive inocula were given intravenously ($2{\cdot}5 \times 10^8$ pock u.), virus was seldom found in the brain. The possibility that virus was effectively removed from the circulation by the lungs so that very little reached the capillaries within the central nervous system was tested. A small inoculum was injected intravenously into each of 56 mice, 28 of which had immediately beforehand been given a traumatizing intracerebral injection of sterile normal saline. At daily intervals, four mice from each group were taken at random and their organs examined and scored for gross abnormalities. Separate

pools were made of the brains, the livers and lungs from each group and these were examined for infective virus.

Three points were noted (Table 6). First, no virus was detected in the brains of mice that had only received virus intravenously, but those that had also been subjected to cerebral trauma showed a steady exponential rise in the concentration of infective virus in the brain from the first day onwards. Secondly, small amounts of virus were detected in the lungs of the former group on the 2nd and 3rd days, whilst in the latter small quantities were detected from the 2nd to the 7th days. Similarly in the livers of the former group a considerable amount of virus was detected on the 2nd day, whilst in the latter virus was much more persistent. Thirdly, the gross appearance of the livers was broadly similar with both groups of mice, but the congestion in the lung was much more intense in the group of mice which had been treated with intracerebral saline. It is interesting that in both groups congestion of the lungs was maximal on the 5th day of experiment, thereafter it disappeared.

Table 6. *Distribution of infective virus in various situations and gross pathological findings after intravenous inoculation of virus in mice, some of which were also given* 0·05 *ml. sterile physiological saline intracerebrally*

	Mice given intravenous virus only					Mice given intravenous virus and 0·05 ml. saline intracerebrally				
	Gross pathology		Content infective virus			Gross pathology		Content infective virus		
	Lung	Liver	Lung	Liver	Brain	Lung	Liver	Lung	Liver	Brain
1	−	±	0	0	0	−	+	0	3	1
2	−	+	7	125	0	−	+	3	8	25
3	−	+ +	3	0	0	−	+	1	14	72
4	±	+	0	0	0	±	+	2	30	106
5	+	+	0	1	0	+ + +	+	6	4	6,670
6	±	+	0	1	0	+ +	+	2	235	1,825
7	−	+	0	0	0	−	+	1	0	47,500

All mice were inoculated with 7×10^3 pock u. strain HFEM. Figures = total infective virus detected (pock u./0·1 ml.) in pools made from four mice. Under gross pathology: −, within normal limits; lung: +, one quarter lungs congested; + +, one-half lungs congested; + + +, three quarters lung congested. Liver: +, pale fatty liver; + +, waxy liver. Signs represent average findings per mouse.

It seems then that virus inoculated intravenously and certainly present in the cerebral vessels did not infect the central nervous system in the absence of trauma. It also appears that virus multiplied in one or more extraneural situations, because although the virus demonstrated in the lung may have been contained in the blood, that in the liver was probably multiplying there, since we had the additional information that perivascular inflammation occurred there. The fact that more virus was regularly found in these two sites when multiplication was occurring in the brain suggests that some at least was due to spill-over from the brain. The congestion of the lung which has constantly been found to be greatest at about the fifth day is of interest and cannot be easily explained.

Evidence excluding blood transport of virus to the central nervous system

Data already given were not sufficient to exclude the possibility that blood played a part in carrying virus to the central nervous system and two series of experiments were made to eliminate it. Attempts were made to determine whether detectable amounts of virus reached the cerebral vessels after peripheral inoculation, and the effect of circulating herpes neutralizing antibody was tested.

Test for virus circulating to the brain. A number of mice were inoculated by the standard procedure on the sole of the left hind foot. At daily intervals, a group of mice was given an intracerebral injection of sterile physiological saline. These were then kept for a further 2 days to provide an opportunity for virus localizing in the brain to multiply and the mice were then killed. The concentrations of infective virus in the brain, the cervical and lumbar cords and the sciatic nerves

Table 7. *Distribution of infective virus in various situations after inoculation of virus intradermally by multiple pressure into the pretreated sole of the hind foot of mice, which were also given 0·05 ml. sterile physiological saline intracerebrally*

Day	Right sciatic nerve	Lumbar cord	Cervical cord	Brain
3	< 0	1·30	0·47	< 0
	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0
4	0·00	< 0	< 0	< 0
	—	> 2·0	> 2·0	< 0
	< 0	0·00	< 0	< 0
	> 2·0	> 2·0	2·00	1·48
5	1·18	< 0	0·00	< 0
	1·53	1·93	1·48	0·30
	1·78	0·78	< 0	< 0
	2·06	1·63	< 0	< 0
6	> 2·0	> 2·0	> 2·0	1·18
	1·64	> 2·0	1·54	0·00
	1·49	> 2·0	> 2·0	> 2·0
7	> 2·0	> 2·0	> 2·0	> 2·0
	> 2·0	> 2·0	1·08	> 2·0
	> 2·0	1·40	1·11	< 0
	> 2·0	> 2·0	> 2·0	> 2·0

Results of inoculating a suspension of strain HFEM containing $2 \cdot 7 \times 10^6$ pock u./ml. by multiple pressure into the sole of the right foot which had been pretreated with 10 % sodium chloride. At intervals the mice were given 0·05 ml. sterile saline intracerebrally and 2 days later they were killed and their organs titrated for infective virus.

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

were then determined. The results (Table 7) suggested that insufficient virus was at any time circulating in the cerebral vessels to infect the brain even in spite of injury to it because infective virus was first detected as usual in the lumbar cord whence it ascended as time went by.

Experiments with neutralizing antiserum. In an attempt to prevent virus reaching the central nervous system via the blood, experiments were done using mice which

had been given large amounts of rabbit herpes-neutralizing antibody. Preliminary experiments showed (1) that mice would tolerate large doses (as much as 1·0 ml.) of undiluted inactivated antiserum intravenously provided that it was administered slowly, and (2) that the neutralizing ability of their serum persisted for long enough to make this experimental approach practicable. This is shown by an experiment in which six mice were given different amounts of antiherpetic rabbit serum intravenously and their sera tested for neutralizing ability after 30 min. and again after 6 days. Two of the mice which were given 1·0 ml. serum possessed sufficient antibody to neutralize 99·5 % and 100 % virus by the method of Burnet & Lush (1939*a*) after 30 min. and after 6 days their sera neutralized 94 and 97 % virus. Two mice which had each received 0·5 ml. serum gave the values 98·5 and 99·5 % after 30 min. and 86 and 88 % after 6 days; whilst with two mice which had been given 0·25 ml. serum, the values were 96·5 and 96·5 % at 30 min. and 77 and 84 % at 6 days. Because the 0·5 ml. dose appeared adequate for our purpose and was well tolerated, this dose was used in the experiments which follow.

Table 8. *Effect upon development of local vesicle and paralysis and upon death of injecting neutralizing antiserum at intervals after intradermal inoculation into the sole of the hind foot which had been pretreated with* 10 % *sodium chloride*

No. days after inoculation at which mice were given 0·5 ml. rabbit antiserum	Local vesicle	Paralysis	Death
1	3, 4, 4	7, 7, 8	–, –, –
2	3, 3, 3, 3	10, 7, 7, 9	–, –, –, –
3	3, 3, 2	7, 7, 8	–, –, –
4	2, 4, 3, 3	5, 8, 7, –	7, –, –, –
5	3, 3, 3, 3	6, 7, 7, 7	7, 13, –, –
No serum given	3, 4, 3, 4	5, 7, 7, 8	–, –, –, –

All mice were inoculated with a suspension containing $2·3 \times 10^7$ pock u./ml. strain HFEM. Figures = day upon which local vesicle, paralysis or death were first noted; –, effect not seen up to 14 days.

Effect of neutralizing antiserum on the development of paralysis

A number of mice were inoculated intradermally by the standard procedure, and at intervals groups of mice were given injections of serum. They were observed over a period of 14 days for the development of lesions and of paralysis. Except possibly for one mouse given serum on the 4th day, there is no evidence that circulating antibody prevented either the formation of local vesicles or the onset of paralysis (Table 8). It is interesting that three of the mice that were given serum on the 4th and 5th days died, although there were no signs of injury to the central nervous system.

Effect of neutralizing antiserum on the distribution of infective virus

A number of mice were inoculated by the standard method and 18 hr. later some were each given 0·5 ml. neutralizing antiserum intravenously; the remainder

which were not so treated served as controls. At daily intervals, three mice from each group were killed and infective virus titrated in the central nervous system and the local sciatic nerve. Despite the circulating antibody, the distribution of virus in serum-treated mice was remarkably similar to that in the controls (Table 9). Taking the results of the two experiments together, it was very unlikely that circulating blood was the vehicle by which herpes virus reached the central nervous system.

Table 9. *Effect of circulating neutralizing antibody upon the distribution of virus in mice examined at intervals after peripheral inoculation by the standard procedure*

	Serum-treated mice				Control mice			
Day	Sciatic nerve	Lumbar cord	Cervical cord	Brain	Sciatic nerve	Lumbar cord	Cervical cord	Brain
1	0·60	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
2	0·90	< 0	< 0	< 0	0·00	< 0	< 0	< 0
	0·00	< 0	< 0	< 0	< 0	0·00	< 0	< 0
	< 0	< 0	< 0	< 0	1·11	< 0	< 0	< 0
3	0·48	< 0	< 0	< 0	1·08	0·30	< 0	< 0
	1·80	0·80	< 0	< 0	0·48	0·78	< 0	< 0
	0·48	< 0	< 0	< 0	< 0	< 0	<0	< 0
4	3·32	3·76	0·95	< 0	1·90	5·60	3·69	1·11
	3·48	4·60	1·60	0·60	4·00	4·30	1·00	0·85
	2·85	3·49	1·70	1·20	1·60	3·87	1·11	0·48
5	3·70	4·03	2·48	1·70	4·00	4·70	3·55	< 0
	2·48	1·89	0·48	< 0	3·67	5·60	5·23	< 0
	< 0	< 0	< 0	< 0	2·78	3·04	0·9	0·00
6	2·00	3·56	2·11	1·89	3·15	1·60	2·78	< 0
	3·76	3·46	2·00	1·75	2·60	3·43	< 0	1·08
	3·52	5·00	2·78	< 0	1·60	4·89	5·15	< 0

All mice were inoculated by the standard procedure with a suspension of strain HFEM containing $2 \cdot 3 \times 10^7$ pock u./ml. Eighteen hours later half the mice were each given 0·5 ml. neutralizing antiserum (rabbit). Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

Role of the lymphatic system

Theoretically, there exists a roundabout pathway by which virus might reach the central nervous system from the periphery by way of the lymphatic vessels (Field & Brierley, 1948). Experiments were therefore carried out to exclude this possibility. A number of mice were inoculated by the standard procedure and at intervals virus was sought in the inoculated foot, the lymphatic glands draining the area, the blood and the sciatic nerve and spinal cord. The points that emerged (Table 10) were first, that virus multiplied in the foot and appeared in the sciatic nerve and spinal cord much as was previously found (Table 5); secondly, that infective virus was demonstrated in the blood in low concentration in some mice but with less regularity than was noted before. Thirdly, only very low concentrations of infective virus were demonstrated in the lymphatic glands. Another

experiment to be reported elsewhere, in which the distribution of herpes virus was noted at short intervals after subcutaneous inoculation of the sole of the foot, showed very little virus reaching the local lymphatic glands. These results suggested that the roundabout route from the periphery to the central nervous system put forward by Field & Brierley (1948) was a most unlikely route for this virus in mice.

Table 10. *Distribution of infective virus in the lymphatic system and nervous system at intervals after inoculation by the standard method*

Day	Foot	Inguinal gland	Popliteal gland	Iliac gland	Sciatic nerve	Lumbar cord	Blood
1	4·90	< 0	< 0	< 0	< 0	< 0	< 0
	4·00	< 0	< 0	< 0	< 0	0·00	< 0
	5·08	< 0	0·00	< 0	0·00	< 0	< 0
2	5·21	< 0	0·30	< 0	0·48	< 0	0·00
	4·84	< 0	< 0	< 0	< 0	< 0	< 0
	5·41	< 0	0·48	0·90	1·52	0·78	0·48
3	5·68	< 0	0·60	0·84	1·93	1·26	0·84
	5·77	< 0	0·30	< 0	2·20	0·70	< 0
	4·97	< 0	< 0	< 0	1·42	1·08	0·84
	4·48	< 0	0·70	0·84	0·84	0·78	0·30

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

Table 11. *Distribution of infective virus at intervals after inoculation in the left foot by the standard procedure, including the homolateral gluteal muscles and the opposite sciatic nerve*

Day	Left sciatic nerve	Left glutei	Lumbar cord	Right sciatic nerve
1	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0
	< 0	< 0	< 0	< 0
2	< 0	< 0	0·00	< 0
	< 0	< 0	< 0	< 0
	1·11	< 0	< 0	< 0
3	1·08	< 0	0·30	< 0
	0·48	< 0	0·78	1·11
	< 0	< 0	< 0	< 0
4	1·90	< 0	4·30	< 0
	4·00	0·48	5·60	< 0
	1·60	< 0	3·87	0·00
5	4·00	2·11	4·70	0·00
	2·78	< 0	3·04	1·15
	3·67	< 0	5·60	< 0
6	3·15	1·65	2·00	1·78
	2·60	3·41	3·43	< 0
	1·60	< 0	4·84	< 0

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

The possibility of invasion by direct extension through the tissues

Very little attention has in the past been paid to the possibility that virus might invade the central nervous system by simple extension through the tissues. To be sure, there is no evidence that such an event occurs with any animal studied, but when working with the laboratory mouse, one is impressed by the nearness of the central nervous system to the periphery. To exclude the possibility that virus might penetrate via the intervening tissues, mice were inoculated by the standard procedure and at intervals infective virus was sought in the infected foot, the sciatic nerve, the muscles overlying it, the lumbar cord and the opposite sciatic nerve. No infective virus was demonstrated in the muscles overlying the sciatic nerve until the 4th day of the experiment, long after it had been demonstrated in the sciatic nerve and in the central nervous system itself (Table 11). It is interesting to note that virus was demonstrated in the contralateral sciatic nerve soon after it had been found in the lumbar cord. It seemed plain that virus was not progressing to the central nervous system by direct extension through the tissues.

The role of the peripheral nerve

Since all the evidence suggested that the peripheral nerve was behaving as a conduit for virus two approaches were tried to reinforce this evidence; (1) observation of the effect of sectioning the sciatic nerve and (2) testing for virus in dorsal root ganglia of infected mice.

Table 12. *Effect of surgical resection of the sciatic nerve upon the ability of infective virus to appear in the central nervous system*

Mice with interrupted sciatic nerve		Control mice	
Foot	Lumbar cord	Foot	Lumbar cord
4·89	< 0	4·71	1·36
4·72	0·85	3·90	2·35
4·10	< 0	4·66	1·40
3·69	< 0	3·59	2·83
4·26	< 0	4·72	2·93

Mice were inoculated by the standard procedure with a suspension of HFEM containing 8×10^7 pock u./ml. Five days later the foot and lumbar cord of each mouse was titrated for infective virus.

Values indicate $\log_{10}$ pock u./0·1 ml. organ extract.

Effect of nerve section

Several preliminary experiments had shown that interruption of the sciatic nerve by surgical section, freezing with carbon dioxide snow and intraneural injection of neutralizing antibody gave equivocal results and in some instances such procedures actually appeared to hasten the appearance of virus in the central nervous system. This might have been due to incomplete interruption of the nerve, to virus multiplying 'through' the resulting inflammatory tissue, or to a combination of both. To overcome both these objections, two groups, each of five mice, were used, one of which was used for control purposes. Mice of the other group

were anaesthetized and their left sciatic nerves exposed. A segment of the nerve 3–5 mm. long was then resected. After suturing the skin, the mice were then left undisturbed for 3 days and except for complete flaccid paralysis of the left hind limbs of the operated mice, all were well. On the 4th day all mice of both groups were given subcutaneous injections of 10% sodium chloride into the sole of the left foot and on the 5th day after the nerve resection, all were inoculated on this site by multiple pressure. Five days later (i.e. 10 days after the operation) all the mice were killed and virus was titrated in the inoculated foot and in the lumbar cord. Infective virus was found in the feet of all mice in similar quantity (3·90–4·89 $\log_{10}$ pock u.) (Table 12); but whereas it was found in the lumbar cords of the control group in all cases (1·36–2·39 $\log_{10}$ pock u.) it was demonstrated in this situation in only one of five mice with sectioned sciatic nerves and then in smaller amount (0·85 $\log_{10}$ pock u.). It was inferred that virus did generally travel to the central nervous system by way of the sciatic nerve and that its passage had been prevented by the resection, but the objections to so simple an explanation will be discussed later.

Table 13. *Distribution of infective virus in the foot, sciatic nerve, dorsal root ganglion and lumbar cord at intervals after inoculation by the standard procedure*

Day	Foot	Sciatic nerve	Dorsal root ganglion	Lumbar cord
1	2·95	< 0	< 0	< 0
2	4·70	< 0	0·18	< 0
3	5·3	< 0	0·74	0·0
4	5·15	1·15	1·85	1·3

Results after inoculation with 2×10^6 pock u. virus. Values given are infective virus per 0·1 ml. $\log_{10}$ pock u.

The appearance of virus in the dorsal root ganglia

It has already been pointed out that the method usually used for taking tissue samples left the dorsal root ganglia *in situ* in the carcass. Attempts were made to remove these structures by straightforward dissection and to titrate them for infective virus along with the sciatic nerves, the lumbar cords and the feet of peripherally infected mice. Accordingly, a number of mice were inoculated by the standard procedure and at intervals mice were killed and tissue samples taken. The dissection was found very difficult and in only four instances were the ganglia successfully removed. However, the results of titrating for infective virus which are shown in Table 13 suggested that the virus had passed centripetally via the sciatic nerve trunk and reached the sensory ganglia before it reached the spinal cord.

DISCUSSION

Some strains of herpes simplex virus are virulent for mice when inoculated intracerebrally whilst others are not (cf. Burnet & Lush, 1939*b*; Florman & Trader, 1947). The ideal host-virus combination for this study would have been one in which one infecting particle was sufficient to cause encephalitis when

inoculated intracerebrally. The strain HFEM must have come close to this requirement for it was clear that for two mouse strains the intracerebral dose of virus required for one LD 50 was close to that required to immunize 50 % of mice so inoculated and that it was of the same order as the pock u. Moreover, the PD 50 when given intraspinally is close to the 50 % immunizing dose.

It is also well established that strains of virus when inoculated intracerebrally may be virulent for mice of any age, but, when inoculated peripherally, they may only be virulent for young mice. This was shown for herpes virus by Andervont (1929), for this and several other viruses by Lennette & Koprowski (1944) and recently it has been confirmed by Johnson (1964*b*). It was not surprising, therefore, that, using mice in the 5th week of life, we should find very few which contracted encephalomyelitis after peripheral inoculation. But using the technique of inoculating intradermally into a patch of oedema resulting from a previous subcutaneous injection of 10 % sodium chloride (Olitsky & Schlesinger, 1941) it was found possible to precipitate virtually 100 % paralysis with suspensions of virus which had readily attainable infective titres. Moreover, by using multiple pressure it was possible to infect mice without causing artificial tensions in the tissues. In fact, the host virus combination would have been nearly perfect had the infection given rise to more clearly defined histological disturbances so that we could have employed another criterion for the movement of virus. This might have been especially valuable because one of the limitations of infectivity titrations was that we did not follow the path of non-infective virus, and possibly virus may have moved intracellularly in a non-infective state. Indeed, Sanders (1953), working with mice, obtained some evidence suggesting that encephalomyocarditis virus travelled in this way.

In several experiments the distribution of infective virus was examined at intervals after peripheral inoculation. Although the high concentrations of infective virus found in one of these (Table 5) were not attained in other experiments, the distributions conformed to a general pattern in which high concentrations of virus were found from the 1st to the 5th days after infection in the inoculated foot, and steadily increasing titres successively in the sciatic nerve, lumbar cord, cervical cord and finally the brain. This strongly suggested that virus invaded the central nervous system at a focal point, possibly related to the roots of the sciatic nerve; it provided a striking contrast to the picture found by Mims (1957) with the Asibi strain of yellow fever virus which gave similar titres in different portions of the central nervous system of the infant mouse after peripheral infection. The pattern did not, however, indicate that virus necessarily passed up the sciatic nerve.

The striking result of resection of the sciatic nerve (Table 12) eliminated the hypotheses of direct extension and of lymphatic transport to the central nervous system. The results obtained were, however, in accord with the idea that virus had passed up the peripheral nerve or that it was blood-borne but had localized in some way by a reflex phenomenon. The latter hypothesis was made most improbable because neutralizing antibody did not interfere with the development of paralysis nor did it prevent the entry of virus to the central nervous system (Tables 8 and 9). Moreover, other evidence (Tables 6 and 7) suggests that virus did not easily penetrate the central nervous system from the blood and that after

peripheral inoculation too little virus was circulating to infect even the traumatized brain. This last finding contrasted with the findings of Burnet & Lush (1938), Sawyer & Lloyd (1931) and Mims (1957) that louping ill virus and yellow fever virus readily infected the traumatized brain of the adult mouse after intraperitoneal inoculation.

Taking the results as a whole, there seems no doubt that herpes simplex virus ascends to the central nervous system of the mouse by way of the peripheral nerve, although the question of how this comes about remains unanswered.

Interest in our problem has recently revived and Johnson (1964*a*, *b*) Platt (1964) and Yamamoto *et al.* (1965) have all examined the behaviour of herpes virus in animals. Of these the work of Johnson (1964*a*) is especially interesting. Using the same strain of virus and descendants of the same colony of mice as were used here, he has made valuable contributions on how herpes spreads in the body mostly using fluorescent antibody techniques. He clearly demonstrates that after subcutaneous inoculation of 4- to 5-day-old mice, virus passes centripetally up nerve fibres to the central nervous system. Only occasional specifically fluorescing cells were found in the local lymph node. No viraemia was detected. But when the virus was introduced by certain other routes there was evidence of haematogenous spread. It is interesting that cells in the peripheral nerves supplying the inoculated area specifically fluoresced, suggesting that the virus was progressing stepping-stone fashion up the nerve. The findings of Yamamoto *et al.* were similar. We found (unpublished observations) supportive evidence for this idea inasmuch as straightforward histological techniques showed chromatolysis and ballooning of nuclei in the Schwann cells of affected sciatic nerves. It remains to be formally demonstrated that these fluorescent cells really contribute to spread by producing infective virus and are not merely silent witnesses of centripetally moving virus in the periaxonal spaces; it is possible, though improbable, that the fluorescence indicates abortive infection in which antigens are formed but not infective virus.

Most of this work was done at the Walter & Eliza Hall Institute whilst holding the British Memorial Fellowship in Virology 1953–4.

SUMMARY

1. The strain HFEM of herpes simplex virus is highly virulent for the 5-week-old mouse when inoculated intracerebrally; the LD50 and the ID50 both being close to the egg infectious unit. It is also virulent when given intraspinally.

2. When inoculated peripherally it seldom causes nervous symptoms in the normal mouse but regularly does so if inoculated into skin which has previously been given a subcutaneous injection of 10 % sodium chloride.

3. After intradermal inoculation of pretreated mice with strain HFEM, the paralysis is preceded by the appearance of infective virus in the blood, the sciatic nerve and the central nervous system. Invasion of the central nervous system is prevented by interruption of the local peripheral nerve.

4. There is no evidence that virus enters the central nervous system by way of the lymphatics or by direct extension through the tissues.

5. After intravenous inoculation virus does not invade the central nervous system unless (*a*) massive inocula are used or (*b*) the brain is traumatized by injecting sterile saline. After peripheral inoculation insufficient virus to infect even traumatized brain seems to circulate in the cerebral vessels. Paralysis and invasion of the central nervous system are not prevented by circulating neutralizing antibody given 18 hours after inoculation.

It therefore appears that invasion of the central nervous system takes place only by way of the peripheral nerve.

REFERENCES

ANDERVONT, H. B. (1929). Activity of herpetic virus in mice. *J. infect. Dis.* **44**, 383.

BEVERIDGE, W. I. B. & BURNET, F. M. (1946). The cultivation of viruses and rickettsiae in the chick embryo. *Spec. Rep. Ser. med. Res. Coun.* no. 256.

BODIAN, D. (1952). A reconsideration of the pathogenesis of poliomyelitis. *Am. J. Hyg.* **55**, 414.

BURNET, F. M. (1950). In Poliomyelitis following inoculations. *Br. med. J.* i, 1004.

BURNET, Sir F. M. (1955). *Principles of Animal Virology.* New York: Academic Press.

BURNET, F. M. & LUSH, D. (1938). Infection of central nervous system by louping ill virus; investigation by quantitative egg membrane technique. *Aust. J. exp. Biol. med. Sci.* **16**, 233.

BURNET, F. M. & LUSH, D. (1939*a*). The inactivation of herpes virus by immune sera. Experiments using the chorioallantoic membrane technique. *J. Path. Bact.* **48**, 275.

BURNET, F. M. & LUSH, D. (1939*b*). Studies in experimental herpes infection in mice using the chorioallantoic technique. *J. Path. Bact.* **49**, 241.

CRAIGIE, J. (1937). Poliomyelitis: virus and experimental infection. *Can. publ. Hlth J.* **28**, 421.

DOERR, R. & VOCHTING, K. (1920). Sur le virus de l'herpès fébrile. *Rev.gén. Ophtal.* **34**, 409.

FABER, H. K. (1953). *The Pathogenesis of Poliomyelitis.* Illinois: Thomas Springfield.

FIELD, E. J. (1953). Pathogenesis of herpetic encephalitis following corneal and masseteric inoculation. *J. Path. Bact.* **64**, 1.

FIELD, E. J. & BRIERLEY, J. B. (1948). The lymphatic connexions of the subarachnoid space. *Br. med. J.* i, 1167.

FIELD, E. J., GRAYSON, J. & ROGERS, A. F. (1949). Circulatory changes in the spinal cord resulting from peripheral stimulation. *J. Physiol., Lond.* **110**, 14*P*.

FIELD, E. J., GRAYSON, J. & ROGERS, A. F. (1951). Observations on the blood flow in the spinal cord of the rabbit. *J. Physiol., Lond.* **114**, 56.

FLORMAN, A. L. & TRADER, F. W. (1947). A comparative study of pathogenicity of four strains of herpes simplex virus. *J. Immun.* **55**, 263.

GOODPASTURE, E. W. & TEAGUE, O. (1923). Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits, *J. med. Res.* **44**, 139.

HABEL, K. & LI, C. P. (1951). Intraspinal inoculation of mice in experimental poliomyelitis. *Proc. Soc. exp. Biol. Med.* **76**, 357.

HORSTMANN, D, M., MCCOLLUM, R. W. & MASCOLA, A. D. (1954). Viraemia in human poliomyelitis. *J. exp. Med.* **99**, 355.

JOHNSON, R. T. (1964*a*). The pathogenesis of herpes virus encephalitis. I. Virus pathways to the nervous system of suckling mice demonstrated by fluorescent antibody staining. *J. exp. Med.* **119**, 343.

JOHNSON, R. T. (1964*b*). The pathogenesis of herpes virus encephalitis. II. A cellular basis for the development of resistance with age. *J. exp. Med.* **120**, 359.

LENNETTE, E. H. & KOPROWSKI, H. (1944). Influence of age on the susceptibility of mice to infection with certain neurotropic viruses. *J. Immun.* **49**, 175.

MARINESCO, G. & DRAGANESCO, S. (1923). Recherches expérimentales sur le neurotropisme du virus herpétique. *Annls Inst. Pasteur, Paris* **37**, 753.

MARTIN, J. K. (1950). Local paralysis in children after injections. *Archs Dis. Childh.* **25**, 1.

MCCLOSKEY, B. P. (1950). The relation of prophylactic inoculations to the onset of poliomyelitis. *Lancet* i, 659.

MELNICK, J. L. (1945). Poliomyelitis in the bloodstream in the experimental disease. *Proc. Soc. exp. Biol. Med.* **58**, 14.

MIMS, C. A. (1957). The invasion of the brain by yellow fever virus present in the blood of mice. *Br. J. exp. Path.* **38**, 329.

NAGLER, F. P. O. (1946). A herpes skin test reagent from amniotic fluid. *Aust. J. exp. Biol. med. Sci.* **24**, 103.

OLITSKY, P. & SCHLESINGER, R. W. (1941). Effect of local edema and inflammation in the skin of the mouse on the progression of herpes virus. *Science, N.Y.* **93**, 574.

PLATT, H. (1964). The local and generalised forms of experimental herpes simplex infection in the guinea-pig. *Br. J. exp. Path.* **45**, 300.

RHODES, A. J. (1953). Recent advances in the study of poliomyelitis with particular reference to prevention. *Br. med. Bull.* **9**, 196.

SABIN, A. B. (1939). Portal of entry and transmission of poliomyelitis virus. *Int. Bull. Econ. med. Res. publ. Hyg.* A**40**, 1.

SANDERS, F. K. (1953). Possible multiplication cycles in neurotropic viruses. The nature of virus multiplication. *2nd Symp. Soc. gen. Microbiol.* p. 297. Cambridge University Press.

SAWYER, W. A. & LLOYD, W. (1931). Use of mice in tests of immunity against yellow fever. *J. exp. Med.* **54**, 533.

WARD, R., HORSTMANN, D. M. & MELNICK, J. L. (1946). The isolation of poliomyelitis from extraneural sources. IV. Search for virus in the blood of patients. *J. clin. Invest.* **25**, 284.

WENNER, H. A. & KAMITSUKA, P. (1957). Primary sites of virus multiplication following intramuscular inoculation of poliomyelitis virus in cynomolgus monkeys. *Virology* **3**, 429.

WILDY, P. (1954). The growth of herpes simplex virus. *Aust. J. exp. Biol. med. Sci.* **32**, 605.

WILDY, P. (1955). Recombination with herpes simplex virus. *J. gen. Microbiol.* **13**, 346.

WRIGHT, G. PAYLING, (1953). Nerve trunks as pathways of infection. *Proc. R. Soc. Med.* **46**, 319.

YAMAMOTO, T., OTANI, S. & SHIRAKI, H. (1965). A study of the evolution of viral infection in experimental herpes simplex encephalitis and rabies by means of fluorescent antibody. *Acta neuropath. (Berl.)* **5**, 288.

Comparison of three ventilating systems in an operating room

By O. M. LIDWELL, I. D. G. RICHARDS* and S. POLAKOFF

Cross-Infection Reference Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9

(*Received* 17 *December* 1966)

INTRODUCTION

There is evidence to support the proposition that surgical wounds can be infected by the sedimenting into them of pathogenic organisms which have been dispersed into the air from persons entering the operating room and from non-sterile materials brought in with the patient (e.g. Blowers, Mason, Wallace & Walton, 1955; Shooter, Taylor, Ellis & Ross, 1956). Some controversy has arisen as to whether downward displacement ventilation, the so-called piston system, might be substantially more effective than other methods of ventilating operating rooms in reducing the risk of contamination of surgical wounds by airborne organisms.

An attempt was made to resolve this controversy by installing alternative ventilation systems in one operating room in such a way that a change could be made from any one to any other in a matter of minutes. It was then possible to examine the differences due to the ventilation systems uncomplicated by the many other differences, for example in the methods of work and in the carrier states of the surgical team, which are inevitably involved in a comparison between different hospitals or even between different operating rooms in the same hospital.

Theoretical considerations, and some evidence from experiments with tracer gases, suggest that in an operating room ventilated by an effective downward displacement system the exposure of the wound to bacterial contamination arising from the activities of persons in the operating room would differ substantially according to the position in the room of the person dispersing the organism and according to whether the major part of the dispersion occurred from the upper or from the lower parts of the body (Lidwell & Williams, 1960). The directed air movements should ensure that contamination dispersed peripherally in the room did not reach the sensitive area over and around the operation wound, and that contamination dispersed at a low level did not rise to this region. On the other hand, the generally low rates of air movement might result in an undue exposure to contamination dispersed from the upper part of the bodies of the surgical team clustered around and leaning over the patient. Some observations on the dissemination of bacteria into the air of a cubicle from volunteers dressed in surgical clothes have been published (Bethune, Blowers, Parker & Pask, 1965). Dispersal from below the waist accounted for two-thirds or more of the total.

By sampling the air simultaneously at the centre of the room, as near as possible

* Present address: Dept. of Social and Preventive Medicine, Institute of Preventive Medicine, The Parade, Cardiff.

to the operation site, and peripherally, near the point at which the ventilating air was discharged from the room, and by relating organisms recovered to the sources from which they had been dispersed, it should be possible to demonstrate the practical importance of the different sites of dispersion and the effectiveness of the ventilation system in protecting the wound from them. Similar observations made with a ventilation system giving a high degree of turbulent mixing of the air throughout the room would not be expected to show any substantial difference between the organisms recovered from the two sampling points whatever the site and location of dispersion.

METHODS

The installation

During alterations to the operating suite at the West Herts. Hospital, Hemel Hempstead, Hertfordshire, ventilation equipment was installed in one of the twin operating rooms in the suite so that it was possible to select, from within the operating room itself, any of three alternative systems of air supply.

The arrangement of the ventilating fittings and some other details of the construction and lay-out of the operating room and suite are shown in Fig. 1.

The three systems were:

System A. Downward displacement. The ventilating air was introduced through six diffusers A 1–A 6 spaced, as far as possible, evenly over the ceiling.

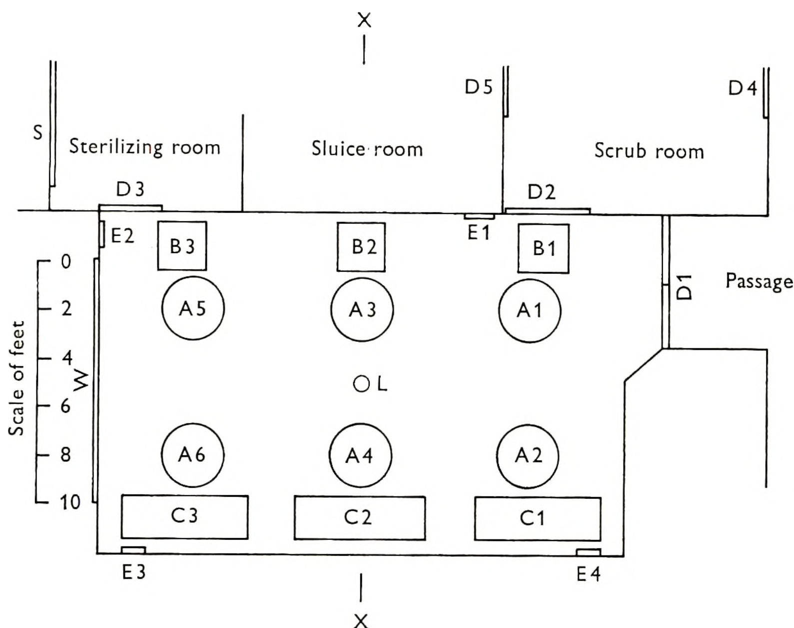
System B. Moderate velocity turbulence. The air entered the room through three grilles B 1–B 3 fitted with directional deflectors. These deflectors were adjusted so as to direct all three air streams towards and above the operating table. The velocity of air movement 1 ft. above the table averaged around 25 ft./min. when the rate of air supply was 1400 ft^3./min.

System C. Low velocity turbulence. The air was introduced vertically downwards at low velocity through three large grilles C1–C3 in the ceiling along one side.

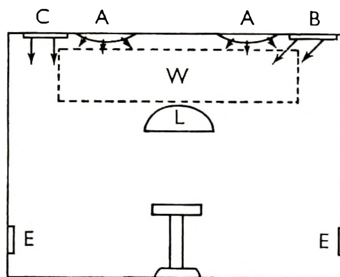
The rate of air supply through any of the three systems could be varied up to a maximum of about 1700 ft.^{3}/min. (with clean filters). The air flow at any time could be read off from an indicating meter connected in the common ductwork for the three systems. Air temperatures were indicated by remote reading thermometers placed in the operating room with sensing elements in the inlet air duct and in one exhaust port, E 3. The majority of the observations were made with air flows within the range 1200–1500 ft^3./min. In this room (volume 3,200 ft^3.) these corresponded to 22–28 air changes per hour.

Initially the six ceiling diffusers fitted for the downward displacement system were circular flush-fitting types, consisting of a set of concentric truncated cones. Diffusers of this type, however, are inherently bistable. According to the disposition of the conical sections the issuing air takes the form either of a downward directed jet, which loses momentum by entrainment from above, or of a sheet flowing horizontally immediately below the ceiling with entrainment from below in the form of a rising air column immediately below the centre of the fitting. Diffusers producing this second pattern of air flow have commonly been employed in 'downward displacement' systems and were initially fitted in this operating

room. Neither pattern of air flow, however, produced anything like a uniform descending air flow or 'piston'. The best approach to uniform air distribution a foot or two below ceiling level, without using an all-over perforated ceiling, is



Plan of operating room
(*a*)



Section of operating room X–X
(*b*)

Fig. 1. Plan and section of the operating room showing the position of the various ventilating fittings. A 1–6, Ceiling diffusers, originally flush fitting concentric conical diffusers (Hospital pattern Airmaster, Fairitt Engineering Co. Ltd.) replaced during the third period of observation by octagonal hemi-elliptical diffusers, 30 in. in diameter, of metal sheet perforated with a regular pattern of $\frac{3}{16}$ in. diameter holes at $\frac{13}{16}$ in. centres; B 1–3, deflecto grilles (Richard Crittall Marine Ltd.), 24 × 22 in., having, one behind the other and at right angles to each other, two sets of individually adjustable blades. C 1–3, deflecto grilles, 62 × 22 in.; E 1–4, exhaust ports, 12 × 12 in., fitted with pressure stabilizers in the form of an adjustable pivoted and weighted flap (Ivo Engineering and Construction Co., Ltd., type 2/S), D 1–5, doors; L, operating lamp; S, autoclaves; W, window, double-glazed, 10 ft. long × 2 ft. high immediately below the ceiling. The blades in grilles B and C were adjusted to direct the ventilating air along the lines indicated by arrows in the figure.

obtained from perforated diffusers of generous dimensions. For this investigation we constructed a set of diffusers each in the form of a hemi-elliptical shell 30 in. in diameter and 8 in. deep assembled from eight quarter elliptical segments pierced with a uniform pattern of $\frac{3}{16}$ in. diameter holes at $\frac{13}{16}$ in. centres. The polygonal shape where these met the ceiling assisted even distribution by promoting entrainment at the angles at ceiling level. With this design and an air supply of 200–250 ft.^{3}/min. to each diffuser substantially even distribution of air was obtained with no residual downward jet or upward induction.

Experiments with tracer gas

Owing to engineering and other difficulties it was only after the plant had been in use for over a year that satisfactory functioning was achieved. Observation with smoke and with a tracer gas (nitrous oxide, see Lidwell, 1960) then showed that even with the redesigned diffuser system the downward displacement arrangement produced only a very imperfect piston-like effect, as is demonstrated by the figures given for system A in Table 1 compared with those to be expected

Table 1. *Air mixing within the operating room with the three ventilating systems*

(Values of the performance index* using nitrous oxide as a tracer gas.)

System	Turbulent air velocity 1 ft. above operating table (ft./min.)	Tracer found over table. Tracer liberated at			Tracer liberated around table at 3 ft. level. Tracer found at		
		5 ft. 6 in. level	3 ft. level	1 ft. 6 in. level	5 ft. 6 in. level	3 ft. level	1 ft. 6 in. level
A	9	3·6	1·1	0·4	0·6	0·9	2·3
B	25	1·6	1·1	0·4	1·3	1·4	*ca.* 2·0
C	9	Not done	1·0	Not done	0·6	0·7	*ca.* 2·8
[P]	Small	Large	Near unity	Very small	Very small	Small	Large
[M]	Large	1·0	1·0	1·0	1·0	1·0	1·0

These measurements were made during the third period of the investigation when good temperature control had been established and the improved diffusers (perforated metal shells) had been fitted to the downward displacement system (system A). The row of the table labelled [P] shows the values that would be expected in an effective downward displacement system. That labelled [M] those which would result from complete turbulent mixing.

* The performance index is the ratio of the quantity of tracer recovered at the sampling point to that quantity that would have been recovered, with the same volume of ventilating air, if air turbulence in the room had been high enough to ensure perfect mixing of air in the room at all times. A low value, therefore, corresponds to less gas reaching the sampling point (Lidwell, 1960).

from an effective downward displacement system. When the tracer gas was liberated at a low level, 1 ft. 6 in. above the floor, in the vicinity of the operating table nearly half as much (performance index 0·4) reached the area immediately above the table as would have been expected if there had been complete turbulent mixing of the air in the room. That there was some layering and restriction of mixing at levels higher than 3 ft. above the floor is indicated by the high value of the performance index over the table (3·6) for tracer liberation at high level in the vicinity of the table and the reduced value (0·6) found at the 5 ft. 6 in. level when tracer was liberated at table level (3 ft.). This experience conforms with our observations

on similar systems installed in other hospitals. Thermal convection currents derived from differences in wall temperatures are usually the dominant factor in determining the patterns of air movement observed.

An air supply of 1200–1500 ft.^{3}/min. evenly distributed, would produce a downward displacement velocity of only about 4 ft./min. in this operating room. The velocities of thermally induced circulation currents are usually substantially greater than this. In this room the outside walls and especially the window, although this was double glazed, were usually one or two degrees centigrade cooler than the central air temperature and produced air movements, including transverse movements a few feet above floor level, which substantially modified the downward displacement pattern.

Table 1 also shows that a certain amount of layering and imperfect mixing also occurred, though to a reduced extent, with the moderate velocity turbulence system B, while the low velocity turbulence system C was in this respect practically indistinguishable from the downward displacement system.

Under all conditions, the ventilation rate, measured by the die-away of the nitrous oxide tracer, was less than the measured air change rate. The difference was usually about 10–15 %. Such a difference can easily arise with the imperfect mixing which is often obtained at high ventilation rates. The increased clearance rate, however, which would result from a true displacement ventilation (Blowers & Crew, 1960) was not observed.

Bacteriological observations

Observations were made during, or in relation to, 323 operating sessions.

Sampling of the operating room air

Air samples were taken simultaneously at a rate of 4 ft.^{3}/min. by two samplers, one placed at the side of the room near to the exhaust outlet E 3, and other sampling from near the operation site (usually within 18 in. of the wound). This second sample was taken through a sterilized horizontal aluminium tube $1\frac{1}{4}$ in. in diameter and either 12, 18 or 24 in. long, which was changed for each operation. The tube was attached to a conical expanding section which was itself connected to the sampler through a right-angle bend, on a 9 in. radius, in a tube 2 in. in diameter. The dimensions of these were calculated to give minimal losses of particles in the connections at the air sampling rate used (Bourdillon & Lidwell, 1948) and tests showed that any losses of this kind amounted to less than 10 %. The samplers, which were specially constructed for this experiment, were modifications of the large slit sampler described by Bourdillon, Lidwell & Thomas (1948) and could take up to 6 samples in sequence automatically on 6 separate plates. The airborne bacteria were collected by impaction on to $5\frac{1}{2}$ in. diameter Petri dishes containing nutrient agar to which 5 % of horse serum and 0·01 % phenolthphalein phosphate had been added. The plates were incubated for 18 hr. at 37° C. and were then exposed to ammonia vapour. Colonies which reddened as a consequence of phosphatase production and had the colonial appearance of *Staphylococcus aureus* were

tested for coagulase production and the coagulase-positive strains were phage-typed. Total colony counts were also made.

Sampling was begun as the first incision was made and was normally concluded when the wound was closed. Individual samples were not collected over a longer period than 20 min. (about 80 ft^3.).

During some sessions, samples were also taken of the bacteria settling on similar plates exposed on the top of the air sampler at the side of the room and as near to the wound as possible. Only total counts of all colonies grown after 24 hr. at 37° C. were made from these plates. Some plates were exposed over the period from wound closure until the time the patient was removed from the room as well as during the operation itself.

Examination of patients and staff

Nasal and skin swabs were taken regularly from all the operating room staff, and from the patients when on the operating table. Members of the staff were swabbed weekly from both nostrils and from the back of the right hand for the first 4 weeks of observations and subsequently at intervals of not more than 1 month. Swabs were taken from the patient's nose, one hand, one axilla, the perineum, and from the skin of the operation site before skin preparation, and from the wound edges before closure at the end of the operation.

Nasal swabs were taken with a cotton wool swab moistened in nutrient broth. Skin swabs were taken with a gauze swab on a $6 \times \frac{3}{4}$ in. wooden spatula moistened with broth.

The swabs were placed in broth for return to the laboratory where they were plated on blood agar and incubated at 37° C. for 24 hr. The broth was incubated overnight and then plated on salt agar which was incubated for 48 hr. Colonies resembling *S. aureus* were tested for coagulase production and the coagulase-positive strains were phage-typed.

It was found possible by examination of the phage-typing patterns to locate probable sources for between 80 and 95 % of the staphylococci recovered from the air at different times during the investigation.

RESULTS

Bacterial contamination of the air

During the first period of observation, which covered 50 operating sessions, the rate of air supply varied substantially. Table 2 shows the degree of bacterial contamination of the air recorded over different ranges of air supply.

The extent of the reduction observed as the air supply was increased accords reasonably well with that to be expected, having regard to the particle size of the airborne bacteria-carrying material. No other analyses have been made of the results obtained during this period.

The second period of observation covered 157 operating sessions. During this time the rate of air supply varied only slightly, between 1200 and 1500 $ft.^3$/min. Temperature control of the incoming air was, however, not good and the flush

fitting conical type diffusers referred to earlier were in use with system A. A third and final period of observations covered 116 operating sessions when temperature control was satsfactory and the hemi-elliptical diffusers had been fitted for use with system A, the downward displacement system.

Table 2. *Effect of ventilation rate on bacterial air contamination*

No. of sessions	Ventilation rate (ft.^{3}/min.)	Mean total count (per ft.^{3})	Rate of dispersal (colony forming units per minute)	Median *Staphylococcus aureus* count (per 100 ft.^{3})
8*	550–900	12·3	$13{\cdot}3 \times 10^3$	1·1
19*	1050–1400	5·5	$8{\cdot}5 \times 10^3$	1·0
23*	1420–1700	6·6	$12{\cdot}4 \times 10^3$	0·9
273†	1375 (mean rate)	5·7	$9{\cdot}6 \times 10^3$	—

* The first three rows of the table are derived from observations made during the first period of the investigation when, owing to mechanical difficulties with the plant, the ventilation rate varied substantially.

† The fourth row gives the mean results obtained during the second and third periods of observation, when the ventilation rate was relatively constant. The results for all three systems have been combined.

Rate of dispersal calculated from the formula

$$\text{rate of dispersal (colony forming units per minute)} = \text{colonies isolated per ft.}^3 \times (\text{ventilation rate} + \text{floor area of room}$$

This assumes complete mixing of the air in the room and a mean settling rate for the airborne particles of 1 ft./min. (see Table 6). The average number of persons present in the operating room during sampling was six so that the mean rate of dispersal (all species of organisms) arising from their activities was about 1600 colony forming units per minute per person.

Over a limited period records were kept of the activity of the operating room staff during sampling periods. The quantitative expression of activity in terms of movement around the room was necessarily somewhat arbitrary and does not include all actions likely to result in dispersal of bacteria, but the results (Fig. 2) show a significant correlation between movement and the level of air contamination.

The performance of the three ventilating systems, during the second and third periods, is compared in Table 3. The figures for total colony count show no significant differences either between the three systems or between the two periods of observation. The figures for *S. aureus* were significantly lower during the third period. This was almost entirely due to the reduced number of occasions when there was a heavy carrier present in the operating room while air sampling was going on. Fewer staphylococci were recovered from the air, during both periods, when the moderate velocity turbulent system, B, was in operation than when the downward displacement system, A, was employed. The differences are not, however, statistically significant.

As a consequence of the fairly high ventilation rate and only moderate dispersion by the staff, the numbers of coagulase positive staphylococci recovered from the air were rather small for detailed breakdown, especially since more than two thirds

of them were probably derived from a single staff carrier (who was usually one of the unscrubbed members of the team). No differences were, however, apparent between the figures obtained with system A (downward displacement) and with system B (moderate turbulence) in respect of the relative contribution of sources situated at the side of the room or around the operating table to the numbers

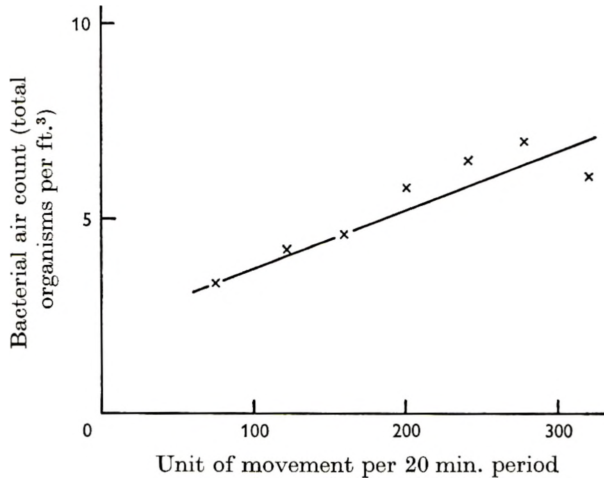


Fig. 2. Relationship between bacterial count in the air of the operating room and staff activity. A unit of movement was recorded each time any person in the operating room moved across the boundary of one of the 18 squares into which the floor of the room was arbitrarily divided. A complete circuit around the operating table produced 12 units of movement

Observations were recorded over periods of 20 min., this being the duration of the individual air samples. There were, on average, with little variation, six persons present in the operating room during these observations. The table is based on movement data collected by Mr D. Wyon whose help we acknowledge with thanks.

Table 3. *Bacterial contamination of the air*

	Second period, 157 operating sessions, about 36,000 ft.^{3} of air sampled				Third period, 116 operating sessions, about 27,000 ft.^{3} of air sampled			
Ventilation system	A	B	C	All together	A	B	C	All together
Average ventilation (ft.^{3}/min.)	1380	1370	1390	1380	1290	1390	1420	1370
Total count (colonies/ft.^{3})								
At operating table	5·7	4·6	5·2	5·2	7·0	6·8	5·2	6·4
At side of room	6·7	6·0	5·1	5·9	6·2	5·9	4·4	5·5
Staphylococcus aureus (colonies/100 ft.^{3})								
At operating table	2·6	1·7	2·8	2·4	1·1	0·6	0·4	0·7
At side of room	3·7	2·2	2·4	2·8	1·1	0·7	0·7	0·8

During the second period temperature control of the ventilating air was poor. During the third period this had been remedied and improved diffusers (perforated metal shells) fitted to the downward displacement system (system A).

recovered from the air over the operating table or from the air leaving the room. With both systems approximately 50 % more colonies were recovered from the table samples than from the side samples when the sources were staff working at the operating table. The side samples similarly contained about 50 % more colonies derived from staff working at the periphery of the room than did the table samples. The results obtained with system C (low velocity turbulence) showed less difference between the sites of sampling. What difference there was was in the opposite

Table 4. *Apparent rate of dispersal of* Staphylococcus aureus *into the operating room (colony forming units per carrier per hour)*

Carrier disperser	Ventilation system A	Ventilation system B	Ventilation system C	All together
Heavy carrier 'X' at operating table	2600	5300	2700	2900
at side of room	8900	3100	4400	5600
Other staff carriers at operating table	210	220	270	240
at side of room	360	270	290	300
Patients	530	110	640	440

The heavy carrier-disperser appeared to be the source of 75 % of the *S. aureus* colonies isolated during the second period of the investigation and of 41 % during the third period or of 70 % during the two periods taken together.

Rate of dispersal calculated from the formula

$$\text{rate of dispersal} = \frac{\text{no. of colonies isolated}}{\text{no. of hours carrier present}} \times \frac{\text{ventilation rate} + \text{floor area of room}}{\text{sampling rate}}.$$

For the average ventilation rate of 1375 ft.^{3}/min., a floor area of 320 ft.^{2} and a sampling rate of 4 ft^3./min.:

$$\text{rate of dispersal (colony forming units per carrier per hour)} = 420 \times \frac{\text{no. of colonies isolated}}{\text{no. of hours carrier present}}.$$

This formula assumes complete mixing into the air of the room and a mean settling rate for the airborne particles of 1 ft./min.

Table 5. *Percentage distribution of sources of* Staphylococcus aureus *recovered from the air*

Sampling site	Source: Patient	Staff at operating table	Staff at side of room	Uncertain or other	Unknown
At table	6	16	51	20	9
At side	5	12	55	20	8

Staff at the operating table comprised the scrubbed staff together with the anaesthetist. The source was uncertain when the same strain was carried by several individuals falling into more than one category.

sense to that found with the other systems. This was, presumably, a consequence of unplanned low velocity air movements. Combining together the samples taken at the operating table and at the side of the room during both the second and third periods of observation a comparison has been made between the three ventilating

systems for those times during which carriers of *S. aureus* were known to be present in the operating room. This has been done by calculating (Table 4) the apparent rate of dispersal of this organism into the room by each carrier. If the ventilating system were more efficient than a fully mixed system this would be reflected in a lower value of the apparent rate of dispersal. It is clear that there is no obvious systematic advantage attributable to any of the three systems tested. It is notable that the one carrier who was also an active disperser was disseminating more than ten times as much as the average carrier. Individual differences of this order of magnitude or greater are not uncommon, a fact which considerably complicates any comparison between different operating suites or any attempt to define an acceptable level of airborne contamination. Table 5 shows the percentage contribution of sources situated in different parts of the operating room to the staphylococcal colonies actually collected in the air samples over the whole period of the investigation. As indicated above, the distribution recorded is dependent on the position in the operating room of the individual carriers encountered.

Table 6. *Numbers of organisms (all species) settling per square foot of exposed surface per minute*

		Ventilation system	
	A (downward displacement)	B (moderate turbulence)	C (low turbulence)
At table, during operation	4·4	6·2	3·1
At side, during operation	3·6	4·5	2·7
At table, after operation	20·2	25·6	16·6
At side, after operation	12·4	21·7	8·8

The numerical similarity between the rate of settling (per ft.²/min.) and the numbers of organisms recovered simultaneously from one cubic foot of air (see Table 3) indicate that the average settling rate of the bacteria-carrying airborne particles approximated to 1 ft./min.

Sedimentation of bacteria from the air

Settling plates were exposed during forty sessions in the third period of observation. As the numbers of coagulase positive staphylococci to be expected on these plates was very small only total counts were recorded.

Table 6 shows the numbers of colonies grown from the plates exposed to direct settling from the air. Any difference between the three systems is again small and not significant although, in these results, the settling rate was consistently greatest with the moderate turbulence system, B, and lowest with the low turbulence system, C. Settling was always greater near the table than at the periphery although the difference was only of the order of 25 % during the operation itself. As would be expected, the number of organisms settling on the plates rose considerably during the activities involved in preparing to remove the patient from the room and this rise was greatest near to the table, where this activity was centred.

Wound contamination and sepsis

Swabs were taken from the wound edges just before closure on 265 occasions. From 255 of these no coagulase positive staphylococci were isolated. From 8 a strain was recovered which was indistinguishable from that carried by the patient. Of the strains isolated from the remaining two, one appeared to be identical with the strain carried by the one active disperser previously referred to, no source could be identified for the other. This is very similar to the four swabs positive for staphylococci from 145 operations three of which were of the type carried by the patient recorded by McNeill, Porter & Green (1961). Burke (1963), on the other hand, who washed out his wounds before closure with sterile saline and cultured the total volume of fluid, recovered coagulase positive staphylococci from 46 out of 50 (92 %). The average number of strains recovered per wound was almost six with an average of more than two colony forming units per strain. His method was clearly much more sensitive and indicates the degree of bacterial contamination of the wound which may occur, almost certainly in this case mainly by the airborne route. It is also apparent, however, that the number of carrier-dispersers present in the operating room during his investigation was substantially larger than in the present instance.

Only six instances of staphylococcal sepsis developing within 14 days of operation were recorded from the 391 operations covered by the period of the investigation. Some cases may have escaped observation but the total numbers were in any case too few for analysis in what was planned as a bacteriological investigation.

DISCUSSION

No evidence could be found from the results of this investigation to suggest that any one of the ventilation systems was significantly superior to the others in protecting a surgical wound from contamination with airborne bacteria. In spite of considerable efforts to improve the design of the diffusers and to control the thermal environment no effective downward displacement air piston was obtained. Although the incoming air was usually a few degrees warmer than the air in the lower part of the room the temperature differences between the walls, and between the walls themselves and the room air temperature were sufficient to generate circulating air movements which were strong enough to prevent the establishment of a slow downward displacement of air. This confirms our observations in other places and is in line with the conclusions of the Heating & Ventilating Research Association's study (Stanley, Shorter & Cousins, 1964). The volume of air supplied was by far the most important characteristic of the ventilation system in controlling the level of airborne contamination. Ventilation systems in which controlled direction of air flow reduces this below the levels obtained with turbulent mixing systems may be obtainable in practice under particular conditions but do not seem to be practicable unless the volumes of air flow relative to the space ventilated are several times greater than those normally envisaged at present. In order to overcome thermal currents, the directed velocities would probably have to reach 10 ft./min. or more. In an operating room similar to the one investigated

by us this would imply a ventilating air supply exceeding 3000 ft.^{3}/min. Experiments with very high flow ventilating systems are in progress in a number of centres in the United States, but in view of the low sepsis rates attainable with ventilating volumes of about 1000 ft^3/min it would seem that much larger rates of air supply could only be justified in exceptional circumstances and that further effort would be better expended in reducing bacterial dispersal by the operating team, for instance by eliminating unnecessary movement and by the use of clothing which is not permeable to bacteria shed from the skin (Blowers, 1963; Bernard, Speers, O'Grady & Shooter, 1965; Blowers & McClusky, 1965). The frequency of glove puncture is also disquieting. During the course of this investigation 15·1 % of the gloves used by the surgeon were found to be punctured by the end of the operation, although there is no evidence that this caused any wound infection. Since there is also good evidence (McNeill *et al.* 1961) that a high proportion of surgical infections are due to strains of organisms carried by the patient before operation finding their way into the wound, further consideration of skin preparation and the method of making the first incision might well be profitable.

SUMMARY

Observations on the bacteriological contamination of the air have been made in an operating room fitted with three alternative systems of ventilation.

These were; A, downward displacement 'piston'; B, moderate velocity turbulent; C, low velocity turbulent.

The volume of the ventilating air supplied was the only characteristic of the ventilation which affected the contamination levels reached during operations. No significant differences could be detected between the three ventilating systems in this respect.

Unavoidable temperature differences in the operating room render it generally impossible to produce effective downward displacement air movement with ventilating air supplies which do not exceed 1500 ft.^{3} per minute over a ceiling area of 300 ft.^{2}.

Great differences were observed between the several carriers of *Staphylococcus aureus* in the extent to which they dispersed this organism into the air when working in the operating room.

Our thanks are due to the N. W. Regional Hospital Board and to their Engineering Department, to the Hospital Secretary, to Mr R. Grainger, Mr P. Stringer and Dr G. A. Matthews, consulting surgeons and pathologist at the hospital, to all the other members of the operating room staff and to the hospital engineer.

REFERENCES

BERNARD, H. R., SPEERS, R., O'GRADY, F. & SHOOTER, R. A. (1965). Reduction of dissemination of skin bacteria by modification of operating-room clothing and by ultra-violet irradiation. *Lancet* ii, 458.

BETHUNE, D. W., BLOWERS, R., PARKER, M. & PASK, E. A. (1965). Dispersal of *Staphylococcus aureus* by patients and surgical staff. *Lancet* i, 480.

BLOWERS, R. (1963). Operating-room practice. In *Infection in Hospitals*, pp. 199–205. Ed. R. E. O. Williams and R. A. Shooter. Oxford: Blackwell Scientific Publications.

BLOWERS, R. & CREW, B. (1960). Ventilation of operating-theatres. *J. Hyg., Camb.* **58**, 427.

BLOWERS, R., MASON, G. A., WALLACE, K. R. & WALTON, M. (1955). Control of wound infection in a thoracic surgery unit. *Lancet* ii, 786.

BLOWERS, R. & MCCLUSKEY, M. (1965). Design of operating-room dress for surgeons. *Lancet* ii, 681.

BOURDILLON, R. B. & LIDWELL, O. M. (1948). Losses of particles during collection. *Spec. Rep. Ser. med. Res. Coun.* no. 262, p. 27.

BOURDILLON, R. B., LIDWELL, O. M. & THOMAS, J. C. (1948). A large slit sampler for air containing few bacteria. *Spec. Rep. Ser. med. Res. Coun.* no. 262, p. 19.

BURKE, J. F. (1963). Identification of the sources of staphylococci contaminating the surgical wound during operation. *Ann. Surg.* **158**, 898.

LIDWELL, O. M. (1960). The evaluation of ventilation. *J. Hyg., Camb.* **58**, 297.

LIDWELL, O. M. & WILLIAMS, R. E. O. (1960). The ventilation of operating theatres. *J. Hyg., Camb.* **58**, 449.

MCNEILL, I, F., PORTER, I. A. & GREEN, C. A. (1961). Staphylococcal infection in a surgical ward. *Br. med. J.* ii, 798.

SHOOTER, R. A., TAYLOR, G. W., ELLIS, G. & ROSS, SIR JAMES PATERSON. (1956). Post-operative wound infection. *Surgery Gynec. Obstet.* **103**, 257.

STANLEY, E. E., SHORTER, D. N. & COUSINS, P. J. (1964). A laboratory study of the downward displacement system of ventilation in operating theatres. *Lab. Rep. Heat. Vent. Res. Ass.* no. 19.

J. Hyg., Camb. (1967), **65**, 207
Printed in Great Britain

Airborne staphylococci in the surgical ward

BY R. E. O. WILLIAMS
Wright-Fleming Institute, St Mary's Hospital Medical School, London, W.2

(*Received* 27 *January* 1967)

The exposure of hospital patients to airborne staphylococci has commonly been estimated by collecting the bacteria from relatively large volumes of air over rather short periods with the use of one or other form of slit-sampler (Report, 1948). The design of the slit sampler was determined largely by the need to collect particles down to about 1–2 μ in diameter, for which collection by sedimentation on to exposed culture plates is very inefficient (Bourdillon, Lidwell & Thomas, 1941), and it therefore employs a high velocity jet of air impinging on the culture plate. While the slit sampler is a very efficient instrument for collecting airborne particles, it has proved difficult to modify it to give really long-period sampling because of the drying effect of the air-stream on the agar plates. Recently it has been shown that only a very small proportion of the staphylococci in the air of hospital wards are associated with particles less than 4 μ in diameter (Noble, Lidwell & Kingston, 1963) and that the median equivalent particle diameter for airborne staphylococci is about 14 μ. For such particles the deficiency of sedimentation as a method of collection is small: the settling rate of 14 μ particles is such that the number contained in 1 $ft.^3$ of air is approximately equal to the number that settle on 1 $ft.^2$ in 1 min. Since with settling plates it is simple to obtain an integrated sample over a considerable number of hours, it seemed worth exploring the use of this method to monitor the staphylococcal content of the air in hospital wards, as has also been reported by Alder & Gillespie (1964). This paper describes a study in a small surgical ward in which an attempt was made to recognize the sources of the airborne staphylococci and to use the method to study cross-contamination between the separate rooms of the ward.

MATERIALS AND METHODS

Almost all the work reported here was done in a 14-bed surgical ward at St Mary's Hospital London between February 1964 and September 1965; the ward is divided into 4 rooms (Fig. 1) and is used mainly for men and women undergoing thoracic and cardiac surgery. The ward has natural window ventilation; the corridor between the rooms leads to a stairway and the stack effect in this generates a flow of air along the corridor and out of the bedrooms in almost all weathers.

A much shorter study was made in a 22-bed open 'Nightingale' ward, using 4 settling plates placed in the 4 quarters of the ward.

Bacteriological methods

Air sampling

Six-inch diameter Petri dishes of nutrient agar containing 5 % horse serum and 0·01 % phenolphthalein phosphate were exposed on brackets fixed to the wall about 5–6 ft. from the floor. During an initial period of 2 months, 3 plates were used to cover the 24 hr.; subsequently 2 plates were each exposed for 12 hr. and later still, when it was found that the counts on the night and day plate were very

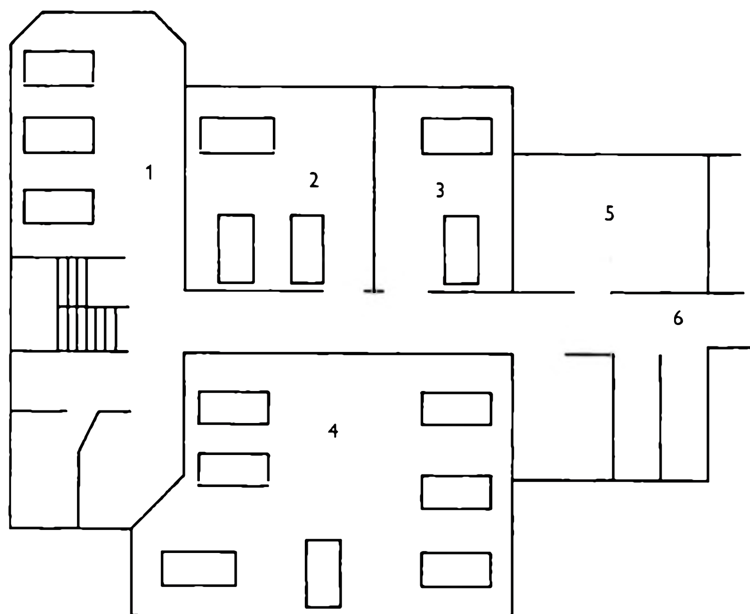


Fig. 1. Diagram plan of ward studied. Rooms 1–4 are bedrooms, 5 is the ward office and 6 is a corridor.

Table 1. *Settling plate counts from different periods of day*

	Period of exposure	*S. aureus* (c.f.u./0·2 ft.²/hr.)
24 Feb.–1 April 1964	6.0 a.m. to 3.0 p.m.	0·99
	3.0 p.m. to 9.0 p.m.	0·75
	9.0 p.m. to 6.0 a.m.	0·66
3 May–25 Sept. 1964	3.0 a.m. to 3.0 p.m.	0·22
	3·0 p.m. to 3.0 a.m.	0·23

Note: By chance there was a very considerable broadcast of staphylococci during the earlier months.

similar (Table 1) a single plate was exposed from 3.0 a.m. to 3.0 p.m. each day (except Saturday and Sunday). For a great part of the survey, plates were also exposed in the ward office to detect dispersal from staff.

The plates were incubated at 37° C. for about 20 hr. and then examined after exposure to ammonia vapour. Colonies showing a bright pink colour were counted

and a sample of them was subcultured for coagulase testing; during most of the study a maximum of 2 or 3 colonies were picked at random from each plate. Coagulase-positive staphylococci were tested for sensitivity to tetracycline, using a 10 μg. disk, and for phage type, using the phages recommended by the International Subcommittee (see Parker, 1962) together with three experimental phages D, 77Ad (now 84) and B5 (now 85).

The term *Staphylococcus aureus* is used for coagulase-positive staphylococci but where 'staphylococci' are referred to without qualification, it is *S. aureus* that is meant.

Carrier surveys

All the 307 patients entering the ward had nasal swabs taken and examined for staphylococci on admission and subsequently once a week; for the first few months the patients also had swabs taken from the skin of the front of the chest. The nursing and medical staff of the ward had hand swabs examined weekly. Nasal swabs from the staff were examined during only the first 2 months.

RESULTS

Numbers of Staphylococcus aureus *in the air*

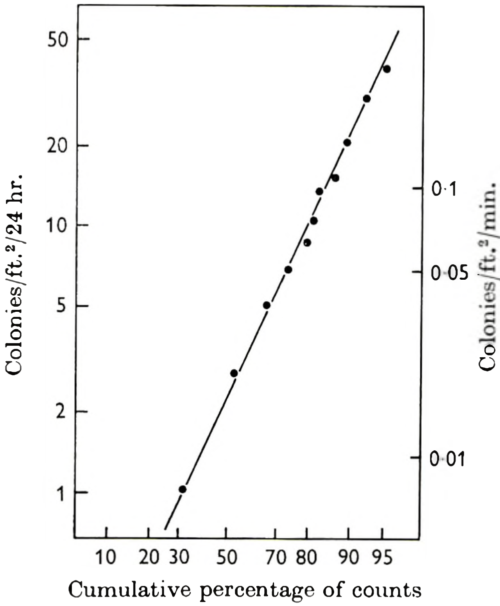
The air counts from all the 4 rooms of the ward for the whole study have been pooled and their distribution is shown in Fig. 2. The counts are plotted logarithmically with a 'probability' scale for the abscissa. The fact that the points fall so close to a straight line indicates that the logarithms of the counts are effectively normally distributed. The median count was 2·6 colony forming units (c.f.u.)/ft.^{2}/24 hr.; 20 % of counts exceeded 11, and 10 % exceeded 24 c.f.u./ft.^{2}/24 hr. The volume of air inhaled by a normal adult in 24 hr. is about 480 ft.^{3}; the median number of staphylococcal particles inhaled by the patients in the ward (on the volume: settling count ratio already noted) was therefore about 4 in 24 hr., with 100 being inhaled on some 3 % of days.

Broadcasts of staphylococci

The day-to-day variation in the air counts was considerable (Fig. 3). At most times the number of staphylococci recovered from the air was less than 25 c.f.u./ft.^{2}/24 hr. but there were 32 periods during which the air count in one or other of the rooms was 50 c.f.u./ft.^{2}/24 hr. or more (Table 2). In most cases the staphylococci found during such 'broadcasts' were almost all of one phage type: in 19 of the 29 shown in Fig. 4, 80 % or more were of one type. Of the total of 32 incidents, 29 appeared to represent dispersal of a single staphylococcus type, 2 were probably made up of two simultaneous dispersals. In the last the peak in the air count was constituted by 4 different types and is not therefore considered further as a 'broadcast'.

Many of the broadcasts lasted only 2 or 3 days and there was little correlation between duration and the number of staphylococci recovered from the air (Fig. 5).

It was possible to recognize a single person, patient or staff, as the probable



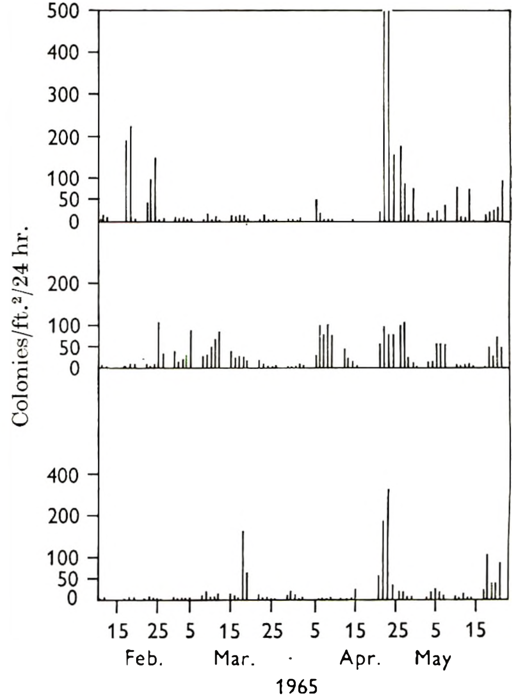


Fig. 2 Fig. 3

Fig. 2. Frequency distribution of counts of *Staphylococcus aureus* in the air of the ward (from Williams, 1966; reproduced by kind permission of the Editor of *Bacteriological Reviews*).

Fig. 3. Daily air counts in 3 of the 4 rooms in the ward (from Williams, 1966; reproduced by kind permission of the Editor of *Bacteriological Reviews*).

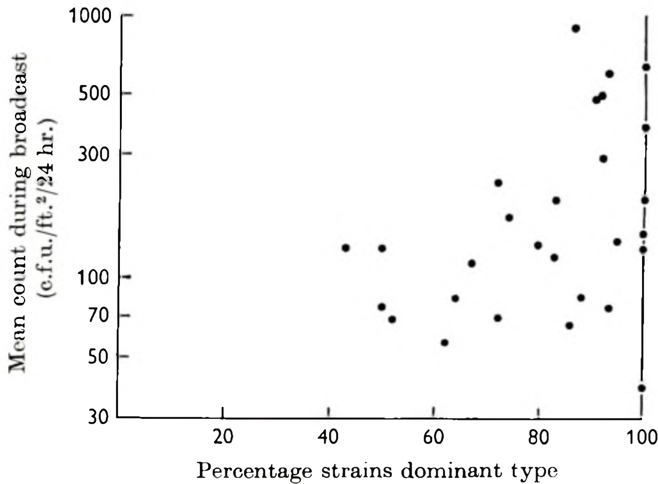


Fig. 4. The prevalence of single phage types of staphylococci during broadcasts (3 broadcasts in which fewer than 5 strains were typed have been omitted)

Table 2. *Broadcasts of staphylococci*

No.	Duration (days)	Type	Tetracycline resistance	Dominant type: No./no. typed	Dominant type: Mean c.f.u./ft.^{2}/24 hr.	Source of broadcast
1	24	29	S	49/54	375	P/N (W)
2	8	80	ND	5/6	165	P/N
3	8	3C/55/71	S	12/12	37	P/N
4	8	6/47/53/54/75/83A	R	14/15	71	P/N
5	5	83A/+	R	12/12	365	P/NW
6	2	*NT*	S	8/9	98	Not found
7a	2	52/7/47/75+	S	4/8	64	Not found
7b		85	R	3/8	55	Not found
8	2	77	S	7/14	39	Not found
9	1	52/52A/80/81	S	10/10	125	P/N
10	17	47/53/54/75/83A/81	S	46/62	123	P/N
11	5	6/7/47/53/54/75/+	S	7/8	84	St/N
12	3	*NT*	ND	12/23	72	(Several possible carriers)
13	3	*NT*	S	5/7	67	(2 possible carriers)
14	2	*NT* (Inh)	R	5/5	240	P/Sp
15	9	75/77	R	7/11	35	P/N
16a	1	*NT* (Inh)	R	1/2	80	P/N
16b		75/77	R	1/2	80	P/N
17	2	6/47/53/54/75/77	S	3/3	113	St/N
18	15	81	R	22/24	260	P/N
19	2	*29*	ND	6/6	145	Not found
20	15	81	R	14/22	53	P and St/N
21	2	*NT*	S	5/7	161	P/N
22	9	80/81/+	R	16/20	93	P and St/N
23	9	85	R	14/16	784	Not found
24	3	81	R	5/6	97	St: several/N
25	9	85	R	13/14	656	P/N
26	3	77/+	S	4/6	75	P/N (W)
27	1	85	R	2/2	1000	P/N
28	29	85	R	40/40	622	P/N (W)
29	8	(*29*)	S	11/12	450	P/N
30	3	4 types	—	6 typed	—	—
31	11	(29)	S	21/22	164	P/N
32	3	52A/6/42E/47/54/75/83A/81/+	S	6/7	55	P/N

Broadcasts 11 and 17; 14 and 16*a*; 15 and 16*b*; 21, 29 and 31; 25, 27, and 28 were each due to single persons; one doctor was probably involved in broadcasts 20 and 24, having been infected during broadcast 18.

Under source: P, patient; St, staff member; N, nasal carrier; W, wound infection; (W), wound infection developing during the course of the broadcast; Sp, sputum infection.

source for 22 of the 33 distinguishable broadcasts, and for 3 others there were 2 or more alternative sources in the ward. Recognition of the sources was often facilitated by the fact that the airborne staphylococci did not appear to spread to any great extent from one room to another (see below). It is possible that members of the staff would have been implicated more often if they had been examined for carriage more effectively, but it was noticeable that, in the broadcasts for which staff were thought to be responsible, staphylococci of the relevant type were found in substantial numbers in all the rooms of the ward and in the ward office (e.g. broadcasts 11 and 17). In only 8 cases was no source definitely recognized and in 2 of these there were actually some nasal carriers present in the ward but the evidence implicating them as the source of the broadcast was poor.

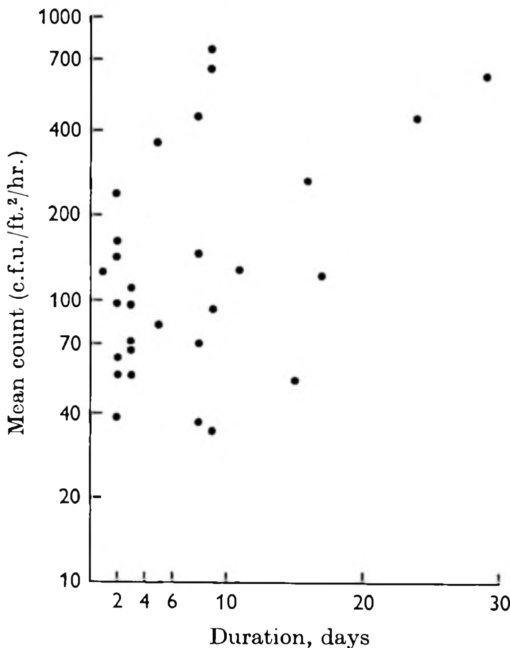


Fig. 5

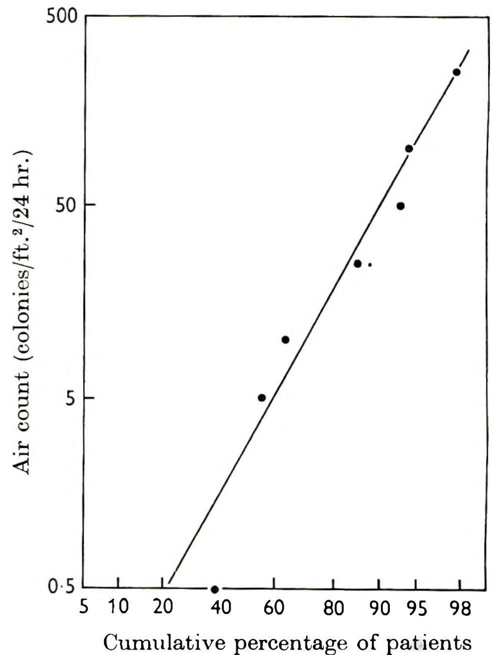


Fig. 6

Fig. 5. Relation of staphylococcal air count during broadcast to duration of broadcast.

Fig. 6. Air counts generated by patients admitted as nasal carriers of staphylococci (from Williams, 1966; reproduced by kind permission of the Editor of *Bacteriological Reviews*).

The broadcasts of tetracycline-resistant staphylococci had a tendency to be greater than those of sensitive strains (Table 2), in that the median values of the mean counts were 150 and 100 respectively.

Twenty of the presumed dispersers were patients and 15 of the 20 were symptomless nasal carriers of the staphylococcus and had no infected lesion of any sort; in none of the remaining 5 was there good reason to think that the infected lesion was the source of the airborne staphylococci. None had a pressure sore. Six individuals (4 patients and 2 doctors) were each involved in more than 1 broadcast and were

between them concerned in 14 of the total of 33 broadcasts. Six of the 16 patients thought to be responsible for broadcasts had acquired their staphylococci in hospital, as had both doctors; 12 of the patients dispersed staphylococci that they carried on admission to hospital and for which there was no indication of acquisition during a previous hospital admission.

During the whole study 85 patients were found, at first examination after admission, to be nasal carriers of a strain of *S. aureus* for which there was no known source already present in the ward. Staphylococci of the same phage type were found in the air of the ward during the ward stay of 53 of these patients; the mean air counts recorded during their stay in the ward are shown in Fig. 6. About 10 % of the patients generated air counts that averaged more than 50 c.f.u./ft.^{2}/24 hr.

In order to assess the sources of airborne staphylococci, the possible sources for each of the various types isolated in each of the 64 weeks were scrutinized; a carrier was recognized among the patients present in the ward for about 30 % of the isolates.

Table 3. *Spread of staphylococci between rooms*

Count in rooms not source of broadcast as % of count in source room	No. of broadcasts	Mean count in source rooms (c.f.u./ft.^{2}/24 hr.)
< 1	8	55, 75, 80, 125, 161, 164, 260, 1000
1–5	5	123, 240, 375, 450, 622
6–10	5	71, 80, 165, 365, 656
11–15	2	35, 37

Incidents during which there were carriers of the broadcast type in non-source rooms are excluded

Spread of airborne staphylococci between the rooms

The simultaneous air sampling in all 4 rooms of the ward enables us to make some assessment of the transfer of airborne bacteria from one room to another.

Of the 33 broadcasts there were 20 in which a single patient was presumed to be the source. The mean count of the index staphylococcus in the 3 rooms other than that harbouring the source is shown in Table 3. In over half the incidents the count in the 'recipient' rooms was 5 % or less of that in the 'source' room. Further examination of the records did not reveal any consistent differences between the individual rooms.

In the open surgical ward there was much less difference between the counts on the 4 plates that were exposed: when a high count was observed on one plate, the count on the plate 20 ft. distant averaged 26 %, and that on the plate 70 ft. distant averaged 11 % of the 'high-count' plate.

Acquisition of the nasal carrier state

The rate of acquisition of *S. aureus* in the nose during hospital stay has frequently been documented and has been suggested as one index of the efficiency with which a hospital layout can protect patients from cross-infection (Parker, John, Emond & Machacek, 1965; Williams, 1966; Lidwell *et al.* 1966). In our ward 6·2 % of

patients were found to harbour tetracycline-resistant staphylococci at first swabbing (0–6 days after admission to the ward); the patients examined after 4 weeks' stay in the ward had a rate of 13·2 % (Fig. 7). The carriage rate for tetracycline-resistant strains on admission is certainly higher than would be found in the normal population because many of the patients had had previous admissions to hospital. It is of some interest that the carrier rate for all staphylococci declined from 31·3 to 26·4 % during the 5 weeks.

Analyses of the potential sources of infection for the 53 patients who acquired typable staphylococci in the nose have been cited elsewhere (Williams, 1966); they showed that staphylococci of the relevant type had been present in the air before the acquisition in 34 cases (64 %).

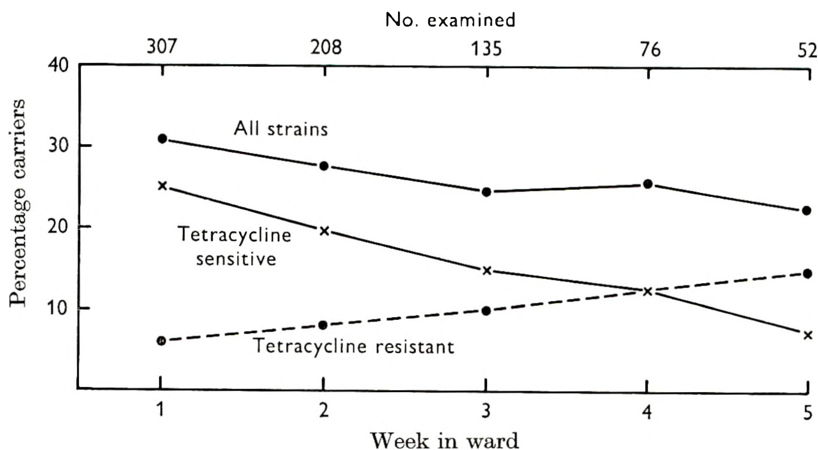


Fig. 7. Nasal carrier rates for *Staphylococcus aureus* after varying periods of stay in the ward. Patients swabbed in week 1 had been in the ward 1–6 days, those swabbed in week 2, 7–13 days, etc.

It has not proved possible to relate the acquisition to the air counts in the ward rooms because patients were moved from one room to another very frequently.

During the survey 4 patients developed wound sepsis due to staphylococci that they apparently acquired while in the hospital. None of these acquisitions occurred during a recognized broadcast and in only one case had the staphylococcus been found in the air prior to the appearance of the wound infection. One other patient who as a carrier was the source of an extensive broadcast (1 in Table 2), developed wound infection with his own strain late in the incident.

DISCUSSION

One of the biggest problems facing those who are concerned with the prevention of cross-infection in a hospital is to find simple ways of predicting trouble; and one of the biggest problems facing those who are asked to advise on the 'hygienic' design of hospital wards is to discover ways in which the efficiency of any particular design can be assessed. Bacterial air sampling seems to have some attractions as an index that might be helpful in attacking both these problems.

There is good evidence that on some occasions, individuals who shed large numbers of *S. aureus* into the environment are the sources of epidemics of infection (e.g. Shooter *et al.* 1958; Blowers, Mason, Wallace & Walton, 1955; Murley, 1965). However most of the dispersers that we recognize among patients in surgical wards seem to be remarkably harmless; in the present study none of the recognized dispersers gave rise to any clinically infected lesions in other people and, more significantly because of the greater number exposed, only one of the 8 very heavy dispersers recorded by Noble (1962) produced an epidemic. In Noble's study this was probably because most of the heaviest dispersers harboured staphylococci lacking in 'virulence' or 'communicability', for the one carrier who caused an epidemic was the only one who had a staphylococcus resistant to penicillin and tetracycline —a pattern very commonly seen in strains that cause epidemics (see Williams, Blowers, Garrod & Shooter, 1966). In the present study the danger of the heavy dispersers was difficult to assess because of the small number of patients exposed to each disperser; half the broadcast strains were tetracycline-resistant, and several of them belonged to phage types known to cause epidemics.

Alder & Gillespie (1964) have suggested that routine examination of ward air, with methods similar to those adopted here, may be useful in indicating potentially dangerous dispersers. Our own results, here and in previous work (Shooter *et al.* 1958; Noble, 1962) suggest that a count of all staphylococci would not be useful because so many dispersers shed what appear to be quite harmless staphylococci. The method might well be useful if it could be made selective for the more virulent staphylococci, but before it is adopted for this purpose we need to have some measure of the frequency with which heavy dispersers of virulent staphylococci recognized in this way subsequently give rise to infections, and what proportion of clinical infections are due to such dispersers.

I have discussed elsewhere (Williams, 1966) the reasons for thinking that the airborne route makes an important contribution to the spread of staphylococci within hospitals and that the non-symptomatic nasal carriage that commonly results is important as the continuing reservoir of hospital staphylococci. One of the desirable features of a ward design should therefore be its contribution to reduction of the airborne spread of staphylococci. This can be detected by air sampling and from this aspect, the frequency of relatively harmless dispersers is an advantage, since it means that there is generally a good supply of staphylococci whose spread can be determined.

For either of these purposes, the use of sedimentation plates seems to have a considerable advantage over the use of mechanical air samplers and, in a long term experiment, few disadvantages.

The rather low rate at which our patients acquired staphylococci in the nose is of interest. It may be compared with the much higher rates recorded in earlier studies at St Bartholomew's Hospital (Williams *et al.* 1962; Noble, Williams, Jevons & Shooter, 1964) where the patients were nursed in open wards, and in recent studies (unpublished) at the Queen Elizabeth II Hospital, Welwyn, where the wards are divided into bays opening widely off a corridor. Recent studies in a subdivided ward at St Bartholomew's Hospital, like the ward studied here used for patients

having thoracic surgery (Lidwell *et al.* 1966), have shown a low acquisition rate similar to ours. In both wards there was a fairly high usage of antibiotics, but, presumably because the subdivision has reduced the rate of transfer of the staphylococci in some way, this has not had the effect of increasing the carrier rate in the way that it did in the patients nursed in open wards (Noble *et al.* 1964).

SUMMARY

The air of a subdivided surgical ward was sampled by the exposure of culture plates for 12 hr. periods 5 days a week.

A number of 'broadcasts' of airborne *Staphylococcus aureus* were observed; many of these were of very short duration. A single person could be recognized as responsible for 22 of the 33 broadcasts; in all cases dispersal seemed to be from carrier sites rather than from infected lesions.

There was little spread of the staphylococci between the 4 rooms of the ward. The frequency of nasal carriage of tetracycline-resistant *S. aureus* increased from 6·2 % in patients examined during their 1st week to 13·2 % in those examined in their 4th week in the ward; the rate for all *S. aureus* declined from 31·3 to 26·4 %.

It is suggested that the long-period exposure of culture plates for the collection of airborne staphylococci may be useful in monitoring some aspects of hospital hygiene.

I am very grateful to Miss B. A. Wimble and Mrs S. Edser for technical assistance; to Sister Jessie Munro and her staff for their support and help in the ward, and to Mr L. L. Bromley for the permission and encouragement to study the patients under his care. The work was supported by a generous grant from the Endowment Funds of St Mary's Hospital.

REFERENCES

Alder, V. G. & Gillespie, W. A. (1964). Pressure sores and staphylococcal cross-infection. Detection of sources by means of settle plates. *Lancet* ii, 1356.

Blowers, R., Mason, G. A., Wallace, K. R. & Walton, M. (1955). Control of wound infection in a thoracic surgery unit. *Lancet* ii, 786.

Bourdillon, R. B., Lidwell, O. M. & Thomas, J. C. (1941). A slit sampler for collecting and counting airborne bacteria. *J. Hyg., Camb.* **41**, 197.

Lidwell, O. M., Polakoff, S., Jevons, M. P., Parker, M. T., Shooter, R. A., French, V. I. & Dunkerley, D. R. (1966). Staphylococcal infection in thoracic surgery: experience in a subdivided ward. *J. Hyg., Camb.* **64**, 1.

Murley, R. S. (1965). Infection in the theatre suite. *Bull. Soc. int. Chir.* **24**, 636.

Noble, W. C. (1962). The dispersal of staphylococci in hospital wards. *J. clin. Path.* **15**, 552.

Noble, W. C., Lidwell, O. M. & Kingston, D. (1963). The size distribution of airborne particles carrying micro-organisms. *J. Hyg., Camb.* **61**, 385.

Noble, W. C., Williams, R. E. O., Jevons, M. P. & Shooter, R. A. (1964). Some aspects of nasal carriage of staphylococci. *J. clin. Path.* **17**, 79.

Parker, M. T. (1962). Phage-typing and the epidemiology of *Staphylococcus aureus* infection. *J. appl. Bact.* **25**, 389.

Parker, M. T., John, M., Emond, R. T. D. & Machacek, K. A. (1965). Acquisition of *Staphylococcus aureus* by patients in cubicles. *Br. med. J.* i, 1101.

Report (1948). Studies in Air Hygiene. *Spec. Rep. Ser. med. Res. Coun.* no. 262.

SHOOTER, R. A., SMITH, M. A., GRIFFITHS, J. D., BROWN, M. E. A., WILLIAMS, R. E. O., RIPPON, J. E. & JEVONS, M. P. (1958). Spread of staphylococci in a surgical ward. *Br. med. J.* i, 607.

WILLIAMS, R. E. O. (1966). Epidemiology of airborne staphylococcal infection. *Bact. Rev.* **30**, 660.

WILLIAMS, R. E. O., BLOWERS, R., GARROD, L. P. & SHOOTER, R. A. (1966). *Hospital Infection: Causes and Prevention.* 2nd ed. London, Lloyd-Luke (Medical Books) Ltd.

WILLIAMS, R. E. O., NOBLE, W. C., JEVONS, M. P., LIDWELL, O. M., SHOOTER, R. A., WHITE, R. G., THOM, B. T. & TAYLOR, G. W. (1962). Isolation for the control of staphylococcal infection in surgical wards. *Br. med. J.* ii, 275.

J. Hyg., Camb. (1967), **65**, 219
Printed in Great Britain

An outbreak of *Pseudomonas aeruginosa* (*pyocyanea*) infection in a Premature Baby Unit, with observations on the intestinal carriage of *Pseudomonas aeruginosa* in the newborn

BY C. H. JELLARD AND GILLIAN M. CHURCHER
Public Health Laboratory Service and Department of Pathology
Plymouth General Hospital, Greenbank Road, Plymouth

(*Received 2 January* 1967)

INTRODUCTION

Pseudomonas aeruginosa (*pyocyanea*) infection may occur sporadically, or in outbreaks. It is well recognized that the newborn, and in particular premature babies, are very susceptible to infection with *Ps. aeruginosa* which may vary from symptomless carriage of the organism to a fulminating septicaemia.

This paper describes an outbreak of infection in a Premature Baby Unit, with predominantly intestinal and respiratory signs, which was caused by a single strain of *Ps. aeruginosa*. The outbreak lasted for 16 months because of the persistent carriage of *Ps. aeruginosa* in the faeces of the babies, many of whom were symptom free.

BACTERIOLOGICAL METHODS

Faeces were cultured on 0·03 % cetrimide nutrient agar (Lowbury & Collins, 1955) and incubated for 18–24 hr. at 37° C. No enrichment media were used.

In environmental investigations, surfaces of equipment were swabbed with cotton-wool swabs which had been moistened with nutrient broth. The swabs were incubated in tubes of broth for 24 hr. at 37° C. and then subcultured on cetrimide agar and incubated for a further 24 hr. at 37° C. Cetrimide agar settle plates were exposed for 1–2 hr. and then incubated for 24 hr. at 37° C. Liquid samples were diluted 1/10 with nutrient broth and incubated for 72 hr. at 37° C. Liquid disinfectants were also tested by the method described by Kelsey & Maurer (1966).

Ps. aeruginosa was identified by the morphology and colonial appearance, the formation of pigment, the characteristic smell and a positive oxidase reaction (Kovacs, 1956). Non-pigmented forms were not found. If there was doubt about the identification, subculture was made, to enhance pigment formation, on Wahba & Darrell's (1965) modification of Sierra's (1957) medium, which was incubated for 24 hr. at 37° C.

Bacteriophage typing of selected strains was carried out at the Cross-Infection Reference Laboratory of the Public Health Laboratory Service.

THE PREMATURE BABY UNIT

Most of the premature babies were delivered in the labour wards or operating theatre of the Maternity Department, and were transferred to the Premature Baby Unit immediately after birth for an average of 3 weeks. Healthy full-term babies went to the maternity wards with their mothers.

For the first 9 months of the investigation the Premature Baby Unit had accommodation for 14 babies; the premises were very overcrowded and unsatisfactory, and there were no isolation facilities. From the end of January 1966, during the latter part of the outbreak, the Premature Baby Unit was transferred to a newly designed modern ward with accommodation for 24 babies, which was divided into cubicles.

In the early weeks of the outbreak the infants' feeds were prepared in the Premature Baby Unit; later the feeds were prepared and terminally sterilized in a central milk kitchen in a different part of the building.

Before use, the inner and outer surfaces of the premature babies' incubators were cleaned with 2·0 % Printol (a clear soluble phenolic fluid); the interior of the incubators was then treated with formaldehyde gas. Bacteriological checks of the incubators showed that the disinfection was effective.

THE OUTBREAK

Between 22 April 1965 and 6 August 1966, 418 babies were admitted to the Premature Baby Unit. The birth weight of nearly all the babies was less than 2500 g. ($5\frac{1}{2}$ lb.). *Ps. aeruginosa* was cultured from the faeces of 99 babies; cultures from 62 of these, taken at random at different times during the outbreak, were typed and with one exception they had the phage-typing pattern 7/16/21/44/68/F8/109/119x/352/1214/M4. There were seven deaths due to *Ps. aeruginosa* infection (Table 1). Infections of nose (2), throat (4), eye (2), ear (2), vagina (2), skin (2), due to *Ps. aeruginosa* also occurred during this period.

As a routine, faeces from all babies are examined bacteriologically every week. The unit had been remarkably free from infection during the year before the investigation. Early in April 1965, *Ps. aeruginosa* was isolated more and more often from the babies' faeces (although selective cetrimide medium was not in use at that time). The significance of this finding was not appreciated until later in the month, when infection in three babies became clinically apparent, one baby developing a skin infection, another a nasal infection and the third a throat infection, which were all due to *Ps. aeruginosa*. At that time, it was suspected that the source of this infection was either an aspirator in the labour ward, which was used for the resuscitation of newborn babies and was heavily contaminated with *Ps. aeruginosa*, or a member of the Premature Baby Unit staff who had a chronic otitis externa, due to *Ps. aeruginosa*. However, in both instances, phage typing showed these strains to be different from the epidemic strain, and the original source of the infection was not discovered.

Table 2 summarizes the positive findings of bacteriological examination of the equipment in the labour wards, maternity operating theatre and Premature Baby

Unit. A total of 210 swabs was taken from different sources, including medicines, hand lotions, soap, distilled water, saline solutions, incubators, babies' feeds and bottles. *Ps. aeruginosa* was isolated on 23 occasions. In spite of the continued faecal

Table 1. *Deaths due to* Pseudomonas aeruginosa *infection*

Case	Date of birth	Sex	Birth weight (g.)	Age at death (days)	Clinical findings	Bacteriological findings	
1	20. v. 65	F, twin	1930	12	Septicaemia	Faeces	†Ep
						Blood culture	†Ep
						Post mortem	
						Meninges	†Ep
						Pericardium	*
2	28. vi. 65	M	1680	10	Respiratory distress syndrome, septicaemia	Throat	*
						Post mortem	
						Pericardium	*
						Bronchi	*
						Meninges	†Ep
3	11. vii. 65	F	1445	11	Septicaemia	Faeces	†Ep
						Post mortem	
						Meninges	†Ep
						Pericardium	*
						Bronchi	*
4	8. i. 66	M	1810	10	Abdominal distension, ileus, laparotomy, gangrene of transverse colon, septicaemia	Blood culture	†Ep
						Mouth	*
						Faeces	*
						Peritoneum	*
5	9. iii. 66	M, twin	1560	11	Respiratory distress syndrome, abdominal distension, septicaemia	Faeces	†Ep
						Post mortem	
						Bronchi	*
						Small intestine	*
						Meninges	†Ep
6	25. iii. 66	F	1470	50	Respiratory distress syndrome, diarrhoea with blood, septicaemia	Faeces	†Ep
7	15. iv. 66	M, twin	1100	13	Respiratory distress syndrome, diarrhoea, septicaemia	Faeces	†Ep

† Ep = epidemic strain of *Ps. aeruginosa*.
* = *Ps. aeruginosa* cultured, but not phage typed.

excretion of *Ps. aeruginosa* during the outbreak the epidemic strain was only isolated twice—from a small mop on 12 October 1965, and from a nail brush on 14 October 1965—in the Premature Baby Unit. Both mop and nail brush were immersed in 2·0 % 'Printol' when sampled. Settle plates failed to show evidence of airborne infection.

In view of the work of Wilson, Nelson, Phillips & Boak (1961), a special

investigation was made of the tap aerators and the outlet of the wash-basins and the sinks. *Ps. aeruginosa* was not isolated from the aerators or taps, but was present in all 36 of the basin and sink outlets tested. The epidemic strain was not found. Each ward had its own predominant basin and sink outlet strain of *Ps. aeruginosa.* Although we could find no evidence to suggest a relationship between the *Ps. aeruginosa* in the basins and sinks and human infection, we agree with Wilson *et al.* that this is a potential source of infection. There is no effective way of sterilizing these outlets and there is a need, in hospitals, for an outlet that can be easily disinfected and made safe.

Table 2. *Sources of* Pseudomonas aeruginosa *from the environment (excluding basins and sinks)*

Maternity operating theatre	2. vi. 65	Rubber tubing in sluice	7/21/68/119x/M4
	18. viii. 65	Floor	N/T
	15. x. 65	Nail brush	N/T
	8. iii. 66	Mop	N/T
Labour ward	20. iv. 65	Aspirator	31
	31. v. 65	Aspirator	7/31/73/119x
	12. vi. 65	Aspirator	7/31/73/119x
	13. viii. 65	Bucket	31
	13. viii. 65	Bucket	31
	14. i. 66	Bucket	N/T
Premature Baby Unit	12. x. 65	Mop in 'Printol'	7/16/21/44/68/F8/109/119x/352/1214/M4*
	12. x. 65	Soapy water in bucket	N/T
	12. x. 65	Mops in 'Printol'	7/53+
	14. x. 65	Nail brush in 'Printol'	7/16/21/44/68/F8/109/119x/352/1214/M4*
	14. i. 66	Bowl	N/T
	14. i 66	Bucket	N/T
	14. i. 66	Mop	N/T
	14. i. 66	Water on floor	68
	14. i. 66	Bucket containing 'Printol'	N/T
Maternity ward	12. x. 65	Aspirator	7/31/73
	12. x. 65	Nail brush	N/T
	14. x. 65	Bucket	31
	17. x. 65	Bottle brush	68+

* = Epidemic strain. N/T = Non-typable.

Examination of faeces

Because of the failure to find a source or reservoir of infection in the environment of the ward or operating theatre, nose and throat swabs and specimens of faeces were obtained from the staff, and the faeces of all babies, both full term and premature, were examined. *Ps. aeruginosa* was not isolated from the staff.

Premature babies

Figure 1 shows the results of a weekly survey of the faeces of the babies in the Premature Baby Unit. Most of those babies who acquired *Ps. aeruginosa* did so during the first 2 weeks of life.

In order to determine the duration of faecal excretion of *Ps. aeruginosa*, 10 premature babies were followed up after they had left hospital, and samples of their faeces were examined at weekly intervals. When, for administrative reasons, this investigation was stopped, five of the babies were still excreting *Ps. aeruginosa* in their faeces 2 months after they were first found to be faecal carriers.

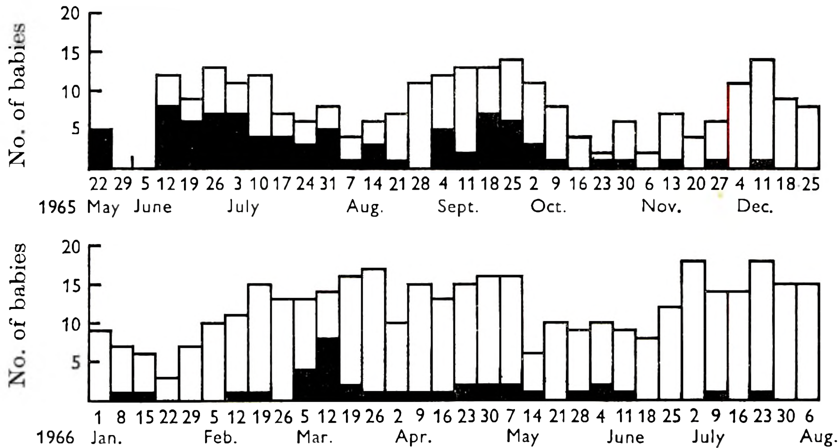


Fig. 1. Weekly isolations of *Pseudomonas aeruginosa* from faeces of babies in the Premature Baby Unit. ■ = *Ps. aeruginosa* isolated from faeces; □ = *Ps. aeruginosa* not isolated from faeces

Table 3. *Monthly examination of faeces of full-term babies in maternity wards*

	1965							1966			
	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	April
Ps. aeruginosa absent	33	33	38	23	39	15	33	34	—	31	18
Ps. aeruginosa present	34	4	11	0	0	1	1	0	—	1	0
Epidemic strain of *Ps. aeruginosa*	20/21	4/4	5/5	—	—	1/1	1/1	—	—	1/1	—

Thirty-three strains of *Ps. aeruginosa* were typed; of these, 32 were the epidemic strain.

Full-term babies

Table 3 shows that, on 2 June 1965, 34/67 normal full-term babies in the Maternity Department wards were excreting *Ps. aeruginosa* in their faeces, many of them in large numbers. Twenty-one strains from these babies were phage typed; 20 belonged to the epidemic strain. All these babies were well and had no symptoms of infection, except one baby who had mild diarrhoea. Monthly bacteriological examination showed a reduction in the faecal carrier rate of these babies which may have been due to improvement in hygiene after the recognition of the infection.

Reservoir of infection and means of spread

Because *Ps. aeruginosa* was not found in the environment, we considered that the babies' faeces were the main reservoir that maintained the infection, for the faeces and nappies were always a prolific source of the organism. The infection was probably spread from baby to baby by contact, by way of the nurses' hands.

DISCUSSION

Many outbreaks of neonatal *Ps. aeruginosa* infection, mainly affecting premature babies, have been reported from the Continent and from the U.S.A., but there have been comparatively few reports from this country. The subject has been reviewed by Alison (1961) and by Paul & Marget (1964).

The infection may affect various parts of the body, such as the respiratory tract, skin, eyes and meninges. Kwantes (1960) described a small outbreak in which middle ear infection predominated, with symptomless intestinal carriage of *Ps. aeruginosa*. The intestinal tract is most commonly affected and outbreaks characterized by gastro-intestinal manifestations, such as feeding disturbances, abdominal distension, diarrhoea, intestinal ileus, haemorrhage, mucosal ulceration and intestinal perforation, have been reported (Schaffer & Oppenheimer, 1948; Bielicka & Dzieniszewska, 1953; Brádaĉ, Procházková & Sedlák, 1954; Neter & Weintraub, 1955; Verger & Bentégeat, 1955; Eggers & Wöckel, 1958; v. Gavallér, 1958; Jacobs, 1964; Bassett, Thompson & Page, 1965).

Ps. aeruginosa is not a usual commensal organism of the intestinal tract in infants. McFarlan, Crone & Tee (1949 and personal communication) failed to find it in the faeces of 385 babies from two maternity units; it has been isolated many times from healthy newborn babies in other hospitals, although sometimes it has caused severe illness. In the outbreak described by Bielicka & Dzieniszewska, 70 babies had severe toxic enteritis; these authors and Linde, Koditz & Kittlick (1962) have postulated a secondary infective factor—possibly a virus or unidentified bacterium—which would enable *Ps. aeruginosa* to act as an intestinal pathogen. Different strains of *Ps. aeruginosa* may vary in pathogenicity, which might account for the variation in severity of different outbreaks. In an outbreak quoted by Verger & Bentégeat (1955), eight babies in a premature baby unit had severe diarrhoea and six of them died from the infection. In the present outbreak, although different non-epidemic strains of *Ps. aeruginosa* were isolated from equipment, wash basins and sink outlets, they were never found to colonize the babies' intestines (or to cause any other infection). This might indicate a difference in the ability of different strains to infect the intestine (with or without symptoms) and that the epidemic strain had an affinity for the intestine. The maturity of the baby might also influence the course of the infection. Linde *et al.* (1962) observed that the incidence of *symptomless* intestinal carriage increased with the age of the baby. In our investigations we found that 34/67 (51 %) full-term babies in the maternity unit were excreting *Ps. aeruginosa* in their faeces; with one exception these babies were symptom free. It was only in the Premature Baby Unit that this organism

behaved as a pathogen. An epidemic of infection was occurring in full-term babies, but because of their greater resistance to infection they were not ill.

Why should *Ps. aeruginosa* be so much more pathogenic to premature than to full-term and older babies, and why is infection becoming more frequent? The following suggestions have been made:

(1) Premature babies may be more susceptible because they lack antibodies. Gitlin, Rosen & Michael (1963) showed that newborn babies were deficient in the bactericidin to Gram-negative intestinal bacilli; the transfer of plasma gamma-globulin fractions across the placenta is selective and 19S gamma-globulin, which includes the gram negative bacillus bactericidin, does not cross the placental barrier and is absent from the plasma of the newborn. The experiments of Jones & Lowbury (1965) who protected mice from *Ps. aeruginosa* septicaemia by passive immunisation with human serum containing antibody to *Ps. aeruginosa*, show the ability of humoral antibodies to control the infection.

(2) Pseudomonas infection in children has become progressively more common since antibiotics were introduced about 15 years ago (Asay & Koch, 1960). The increase of infections has been attributed to the use of antibiotics which suppress the antibiotic-sensitive commensal organisms and favour the selection of resistant species such as *Ps. aeruginosa.* In the early stages of the outbreak, many of the premature babies were having prophylactic treatment with penicillin and streptomycin, which may have made them more susceptible to infection.

(3) Hoffman & Finberg (1955) suggested that the high humidity in the babies' incubators favoured the multiplication of *Ps. aeruginosa.*

(4) The incidence of *Ps. aeruginosa* infection may still be rising because of the survival of more premature babies with modern methods of treatment and intensive care.

The next point to consider is the source or reservoir of the organism. In other studies various sources have been incriminated such as polluted milk (Hunter & Ensign, 1947); contaminated suction apparatus (Bassett *et al.* 1965; Rubbo, Gardner & Franklin, 1966) and contaminated babies' incubators (Barrie, 1965). Verger & Bentégeat (1955) give evidence that the origin of a severe outbreak of infection in their Centre for Premature Babies was a baby who came from another hospital and developed a severe generalized skin infection due to *Ps. aeruginosa* soon after admission to the Centre. In at least three previous outbreaks, the commonest source of *Ps. aeruginosa* was the babies' faeces. Bielicka & Dzieniszewska found 125/170 (73 %) babies were excreting *Ps. aeruginosa* in the faeces and 98 were symptom-free; in Neter & Weintraub's study 71/143 (50 %) of the babies had *Ps. aeruginosa* in their faeces and most of them were healthy; *Ps. aeruginosa* was cultured from the faeces of 17/21 babies in Verger & Bentégeat's epidemic. The reservoir of infection in the present outbreak was also the babies' faeces, the most probable path of infection being by way of the nurses' hands—the organisms not always being removed by soap and water—to infect another baby during feeding, possibly by contamination of the teats (Florman & Schifrin, 1950; McLeod, 1958; Primavesi, 1964).

There are striking similarities between this outbreak in premature babies and

an epidemic of *Ps. aeruginosa* septicaemia in irradiated mice and rats, described by Wensinck, Van Bekkum & Renaud (1957) in which infection was caused by contamination of drinking devices by faecal excreters of *Ps. aeruginosa*; the epidemic was controlled by elimination of the faecal carriers and sterilization of the drinking bottles.

The type of outbreak described in this paper is difficult to control. The staff should be fully aware of the possible means of spread of the infection and should carry out the every-day routine preventive procedures with meticulous care. This is particularly important because of the unreliable action of liquid disinfectants on *Ps. aeruginosa*. The P.H.L.S. committee report (1965) on the use of liquid disinfectants in hospitals, advises that physical methods (heat) be used whenever possible, because of the uncertain action of these disinfectants. Kelsey & Maurer (1966) showed how inadequate these disinfectants may be in general use; in different hospitals, vegetative organisms were recovered from buckets and jars which contained various phenolic disinfectants. Our findings also expose these drawbacks, which are all the more obvious when pseudomonads—which are more resistant to disinfectants than most vegetative organisms—are present. Brushes and mops should not be used, unless they are disposable or can be sterilized (by heat) after every use. If the staff rely only on disinfectants, they may wrongly assume that materials are sterile.

Ideally, the following measures are recommended to control the spread of infection:

(1) The babies should be nursed with isolation precautions in separate cubicles.

(2) Nurses who change nappies should not feed babies.

(3) Dirty nappies should be changed with extreme care and put directly into disposable polythene bags.

(4) Disposable gloves should be worn whenever the baby is handled, because it is difficult to ensure that the hands are completely free of *Ps. aeruginosa* after contamination, owing to the resistance of *Ps. aeruginosa* to soap and water and to disinfectants.

(5) To exclude *Ps. aeruginosa*, regular bacteriological screening tests should be made on the faeces of all premature babies, using a selective culture medium.

These procedures are time-consuming, and shortage of staff may make it difficult for them to be properly carried out.

Because so many of the infected babies are free of symptoms, the results of measures to control infection are not clinically apparent, and it may be difficult to keep the staff enthusiastic about the strict discipline which is necessary. The gravity of the situation, where *Ps. aeruginosa* is spreading silently through a premature baby unit, may not be appreciated until serious infection and deaths occur. 'Il faut redouter le pseudomonas'—pseudomonas must be feared (Alison, 1961)—and everyone should be fully aware of these often hidden but potentially dangerous infections of premature babies in hospitals.

SUMMARY

An outbreak of infection in a Premature Baby Unit due to a single strain of *Pseudomonas aeruginosa* is described. The outbreak lasted for 16 months, during which this organism was isolated from the faeces of 99/418 premature babies, many of them being symptomless carriers. There were seven deaths due to the infection. The source of the epidemic was not found. The main reservoir of infection was the babies' faeces. Though many full-term babies were symptomless carriers of the epidemic strain of *Ps. aeruginosa*, they were not ill.

Measures for control of the outbreak, and the aetiology of neonatal intestinal infection with *Ps. aeruginosa* are discussed.

We wish to thank Dr J. N. Montgomery for his help and for giving us access to his clinical notes.

We are indebted to Dr M. T. Parker and Mrs E. A. Asheshov of the Cross-Infection Reference Laboratory, Colindale, for phage-typing numerous cultures of *Ps. aeruginosa* and for helpful discussion.

REFERENCES

ALISON, F. (1961). Infections à *Pseudomonas aeruginosa* chêz le nouveau-né et le nourrisson. *Actual. pédiat.* 3rd ser. p. 321.

ASAY, L. D. & KOCH, R. (1960). Pseudomonas infections in infants and children. *New Engl. J. Med.* **262**, 1062.

BARRIE, D. (1965). Incubator-borne *Pseudomonas pyocyanea* infection in a newborn nursery. *Archs. Dis. Childh.* **40**, 555.

BASSETT, D. C. J., THOMPSON, S. A. S. & PAGE, B. (1965). Neonatal infections with *Pseudomonas aeruginosa* associated with contaminated resuscitation equipment. *Lancet* i, 781.

BIELICKA, I. & DZIENISZEWSKA, L. (1953). Zakazenie paleczka ropy blekitnej u wcześniaków. *Polski Tygod. lek.* **8**, 1413.

BRÁDAČ, O., PROCHÁZKOVÁ, R. & SEDLÁK, J. (1954). Epidemie smrtelných průjmů novorozenců s nálezem *Pseudomonas aeruginosa* (*B. pyocyaneus*). *Pediat. Listy* **9**, 229.

EGGERS, H. & WÖCKEL, W. (1958). The clinical picture and pathology of pyocyaneus infection in premature and full term neonates. *Germ. med. Mon.* **3**, 45.

FLORMAN, A. L. & SCHIFRIN, N. (1950). Observations on a small outbreak of infantile diarrhoea associated with *Pseudomonas aeruginosa*. *J. Pediat.* **36**, 758.

GAVALLÉR, B. v. (1958). Veränderungen durch Pyocyaneus bei Neugeborenen. *Verh. dt. Ges. Path.* **41**, 213.

GITLIN, D., ROSEN, F. S. & MICHAEL, J. G. (1963). Transient 19S gamma-globulin deficiency in the newborn infant and its significance. *Pediatrics, Springfield* **31**, 197.

HOFFMAN, M. A. & FINBERG, L. (1955). Pseudomonas infections in infants associated with high humidity environments. *J. Pediat.* **46**, 626.

HUNTER, C. A. & ENSIGN, P. R. (1947). An epidemic of diarrhoea in a newborn nursery caused by *Pseudomonas aeruginosa*. *Am. J. publ. Hlth.* **37**, 1166.

JACOBS, J. (1964). The investigation of an outbreak of *Pseudomonas pyocyanea* infection in a pediatric unit. *Post-grad. med. J.* **40**, 590.

JONES, R. I. & LOWBURY, E. J. L. (1965). Susceptibility of man to *Pseudomonas aeruginosa*. *Lancet* ii, 623.

KELSEY, J. C. & MAURER, I. M. (1966). An in-use test for hospital disinfectants. *Mon. Bull. Minist. Hlth* **25**, 180.

KOVACS, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, Lond.* **178**, 703.

KWANTES, W. (1960). An outbreak of acute otitis media in a maternity hospital due to *Pseudomonas aeruginosa*. *Mon. Bull. Minist. Hlth* **19**, 169.

LINDE, K., KODITZ, H. & KITTLICK, M. (1962). Untersuchungen zur Frage der Säuglingsenteritis durch *Pseudomonas aeruginosa*. *Mschr. Kinderheilk.* **110**, 62.

LOWBURY, E. J. L. & COLLINS, A. G. (1955). The use of a new Cetrimide product in a selective medium for *Pseudomonas pyocyanea*. *J. clin. Path.* **8**, 47.

McFARLAN, A. M., CRONE, P. B. & TEE, G. H. (1949). Variations in bacteriology of throat and rectum of infants in two maternity units. *Br. med. J.* ii, 1140.

McLEOD, J. W. (1958). The hospital urine bottle and bedpan as reservoirs of infection by Pseudomonas. *Lancet* i, 394.

NETER, E. & WEINTRAUB, D. H. (1955). An epidemiological study on *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) in premature infants in the presence and absence of infection. *J. Pediat.* **46**, 280.

PAUL, R. & MARGET, W. (1964). *Pseudomonas aeruginosa* infections (pyocyaneus sepsis) in infancy. *Germ. med. Mon.* **9**, 119.

PRIMAVESI, C. A. (1964). Pyocyaneus-Hospitalismus auf einer Neugeborenenstation. *Dt. med. Wschr.* **27**, 1303.

PUBLIC HEALTH LABORATORY SERVICE COMMITTEE ON TESTING AND EVALUATION OF DISINFECTANTS (1965). Use of disinfectants in hospitals. *Br. med. J.* i, 408.

RUBBO, S. D., GARDNER, J. F. & FRANKLIN, J. C. (1966). Source of *Pseudomonas aeruginosa* infection in premature infants. *J. Hyg., Camb.* **64**, 121.

SCHAFFER, A. J. & OPPENHEIMER, E. H. (1948). Pseudomonas (Pyocyaneus) infection of the gastro-intestinal tract in infants and children. *Sth. med. J., Nashville* **41**, 460.

SIERRA, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of contact between cells and fatty substrates. *Antonie van Leeuwenhoek* **23**, 15.

VERGER, P. & BENTÉGEAT, J. (1955). Une épidémie d'entérite à bacille pyocyanique (*Pseudomonas aeruginosa*) observeé dans un centre de prématurés. *Nourrisson* **43**, 141.

WAHBA, A. H. & DARRELL, J. H. (1965). The identification of atypical strains of *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **38**, 329.

WENSINCK, F., VAN BEKKUM, D. W. & RENAUD, H. (1957). The prevention of *Pseudomonas aeruginosa* infections in irradiated mice and rats. *Radiat. Res.* **7**, 491.

WILSON, M. G., NELSON, R. C., PHILLIPS, L. H. & BOAK, R. A. (1961). New source of *Pseudomonas aeruginosa* in a nursery. *J. Am. med. Ass.* **175**, 1146.

J. Hyg., Camb. (1967), 65, 229
Printed in Great Britain

Pseudomonas aeruginosa respiratory tract infections in patients receiving mechanical ventilation*

By IAN PHILLIPS
St Thomas's Hospital Medical School, London, S.E. 1†

(*Received* 1 *February* 1967)

It has been suggested that *Pseudomonas aeruginosa* respiratory tract infection in patients receiving mechanical ventilation arises from contaminated ventilators (Phillips & Spencer, 1965). Efforts to overcome the problem—sterilizing the machines (Bishop, Potts & Molloy, 1962; Sykes, 1964; Alder, Brown & Gillespie, 1966) and the use of filters (Bishop, Roper & Williams, 1963; Bishop, 1966)—have been based on this assumption. The alternative explanation, that the machines might become secondarily contaminated by patients infected by some other, unknown route, has not been excluded however.

This paper describes a study undertaken to determine the direction of spread of *Ps. aeruginosa*—machine to patient or patient to machine. It involved frequent, simultaneous sampling of the flora of the patients' respiratory tracts and of the ventilators in use on them, with the pyocine typing, and in some cases phage typing, of strains of *Ps. aeruginosa* isolated.

INVESTIGATIONS AND METHODS

The patients

All patients requiring tracheostomy and mechanical ventilation (except those recovering from cardiac surgery) during a period of just over one year starting in January 1965, in St Thomas's Hospital, were studied—18 patients in all. The diagnosis, ward and duration of mechanical ventilation for each patient are listed in Table 1. From the time of tracheostomy, swabs were taken at least daily from tracheostomy wounds, and samples of endotracheal aspirate were obtained at similar intervals. In addition, a stool sample or rectal swab was obtained from as many patients as possible before tracheostomy or soon after.

The mechanical ventilators (*Smith-Clarke, Cape Engineering Company*)

Swabs were taken from water-bath humidifiers and the tubing connecting patient to ventilator, between the water-bath humidifier and patient, before use and during use, at daily intervals at least, until contamination with *Ps. aeruginosa* was detected, when the machine was replaced.

After use the ventilators were disinfected by raising the temperature of the water in the humidifiers to boiling, and boiling tubing and metal connectors removable from the machine. The fixed parts of the machine were merely cleaned.

* This paper formed part of a Thesis for the degree of M.D., for the University of Cambridge.

† Present address: Department of Medical Microbiology, Makerere University College, Kampala, Uganda.

Occasionally ventilators were disinfected by the formalin method of Sykes (1964), but facilities were not available for the routine application of this method.

Water-bath humidifiers were left empty until just before use, when they were filled with sterile distilled water and heated to a temperature of about 44° C. In the course of the investigation distilled water was replaced by a solution of chlorhexidine digluconate (pure Hibitane, I.C.I.), 1/5000 in sterile distilled water.

The environment

From time to time, intensive sampling of all parts of the moist environment, and of bed-linen and floor dust, was carried out.

Table 1

Category	Case no.	Diagnosis	Ward	Duration of ventilation (days)	Infection time (days after tracheostomy)	Pseudomonas type from: Patient	Pseudomonas type from: Ventilator	Days between contamination and infection*
Already infected	2	Cor pulm.	MM 1	35	—	S^{II}	—†	—
	3	Cor pulm.	MM 1	42	—	S^{I}	—†	—
Becoming infected	1	Cor pulm.	MM 1	42	26	B	—‡	+26
	4	Post-op.	MS 2	1½	4	S^{I}	—†	?
	5	Cor pulm.	MM 1	28	2	S^{I}	S^{I}	+2
	6	Polyneur.	FM 2	11	3	S^{I}	S^{I}	−3
	7	Post-op.	FM 3	1	1	S^{I}	S^{I}	+1
	8	Post-op.	ICU	10	2	S^{I}	S^{I}	−2
	10	Post-op.	ICU	11	5	S^{I}	S^{I}	+1
	11	Diabetic coma	ICU	6	3	Z	Z	+3
	12	Tetanus	ICU	28	10	S^{I}	Not contam.	—
	13	Barbit. poisoning	ICU	2	6	S^{III}	Not contam.	—
	14	Cor pulm.	ICU	14	5	D	D	+1
	15	Flail chest	ICU	14	5	S^{I}	S^{I}	+3
Not infected	9	Cor pulm.	ICU	28	—	Not infect.	Not contam.	—
	16	Cor pulm.	ICU	11	—	Not infect.	Not contam.	—
	17	Mercury poisoning	ICU	3	—	Not infect.	Not contam.	—
	18	Flail chest	ICU	21	—	Not infect.	Not contam.	—

Cor pulm. = cor pulmonale. Post-op. = post-operative. Polyneur. = polyneuritis. Barbit. = barbiturate.

* + = ventilator contaminated before patient infected; − = patient infected before ventilator contaminated.

† Not studied. ‡ lost before typing.

Bacteriological methods

Isolation and identification of Ps. aeruginosa

Sputum and endotracheal aspirate were homogenized by treatment with buffered pancreatin (Oxoid). All samples were cultured on blood agar, and in 0·03 % cetrimide in nutrient broth, subcultured after 24 hr. at 37° C. to blood agar.

Ps. aeruginosa was usually identified by its well-known typical colonial form and odour, and the identification was confirmed by the demonstration of pyocyanin production on enhancement medium (Pseudomonas Agar P, Difco). Strains with less typical colonial forms were identified by the following sequence of tests: examination of colonies on blood agar in ultra-violet light; Gram stain; oxidase test (Gaby & Hadley, 1957); oxidation of glucose (Hugh & Leifson, 1953); and pyocyanin production on enhancement medium. The identity of the single non-pigmented strain in this series was confirmed by three further tests—for gluconate oxidation (Haynes, 1951); arginine dihydrolase activity (Thornley, 1960); and growth in nutrient broth at 42° C. in three successive subcultures (Haynes & Rhodes, 1962).

Pyocine typing

The method described by Darrell & Wahba (1964) was used with a slightly modified set of indicator strains (Wahba, 1964) supplied by Dr M. T. Parker of the Central Public Health Laboratory, Colindale.

Phage typing

A number of strains with identical pyocine typing patterns were phage typed by Mrs E. H. Asheshov of the Central Public Health Laboratory, Colindale.

RESULTS

The patients

The eighteen patients studied may be divided into three groups—those who were already infected when mechanical ventilation was started (two patients, both with chronic bronchitis and cor pulmonale), those who became infected during treatment (12 patients), and those who never became infected (four patients). These groups are listed separately in Table 1.

The time taken for infection to develop varied from 1 to 26 days, but in all except two cases was less than 1 week.

Only one patient out of seven examined was found to be a faecal carrier of *Ps. aeruginosa* before treatment started: she subsequently developed respiratory tract infection, but the organism had a different pyocine typing pattern from that from the faeces.

The ventilators

Bacteriological study of the ventilators was possible in 15 of the 16 cases in which the patient was not already infected when tracheostomy was performed.

Before the use of chlorhexidine, 37 % of 125 samples from humidifiers contained

Ps. aeruginosa, but after its use, all of 111 samples were sterile. *Ps. aeruginosa* was found in 14·5% of 355 swabs taken from respirator tubing.

In six of the ventilators *Ps. aeruginosa* was never isolated from any part: these included the machines used on the four patients who did not become infected. In the nine cases where contamination was detected *Ps. aeruginosa* was isolated from the ventilator before the patient in seven, and from the patient before the machine in two (Table 1).

The environment

There was frequent contamination with *Ps. aeruginosa* of moist sites, e.g. drains, soap, nailbrushes, ointments, distilled water. Sheets in use on infected patients and floor dust close to these patients, were also often contaminated (Table 2).

Table 2. *Environmental studies*

Site	No. of samples	% positive for *Ps. aeruginosa*	Pyocine types
Respirator tubing	355	14·5	As patients
Water-bath humidifiers	125	37	As patients
Water-bath humidifiers plus chlorhexidine, and nebulizers	111	0	—
Sheets—Infected patients	20	50	As patients
Non-infected patients	20	0	—
Floor dust—Infected patients	20	75	As patients
Non-infected patients	20	0	—
Air (Slit-sampler)	Numerous	0	—
Ointments	16	20	Miscellaneous
Baths, sinks, soap, nail-brushes	Numerous	Frequent	Miscellaneous
Boiled catheters for endotracheal suction	25	0	—
Anaesthetic equipment	Numerous	0	—
Distilled water	6	50	Miscellaneous

Typing results

Pyocine typing

The commonest pyocine inhibition pattern was one not described by Darrell & Wahba: it resembled their type D except that indicator strain B 10 was consistently inhibited. It was labelled type S. Other patterns seen were Darrell & Wahba's types B and D, and inhibition of indicator strain 577 alone, labelled by us type Z.

Nine of the patients who became infected were found to have strains of type S: the ventilators used in these cases had type S organisms in six instances, while two were not contaminated and one was inadvertently not studied. Of the three patients who did not have type S strains, one had type B, another type D, and the third type Z. In the last two cases, the ventilators had organisms with identical patterns; in the first the strain was lost before typing.

Pyocine types of organisms from sheets and dust were the same as those of the organisms isolated from the respective patients. In contrast, organisms isolated from other sites were of a variety of pyocine types, distinct from those of the patients' organisms.

Phage typing

Pyocine type S was shown to include organisms with three different phage-typing patterns, which were labelled S^{I}, S^{II}, and S^{III} (Table 3).

Type S^{II} was isolated from one of the patients who was already infected (case 2) and type S^{III} from a patient whose ventilator was not shown to be contaminated (case 13). The remainder were S^{I}.

On the basis of pyocine typing and phage typing, 15 strains out of 22 isolated from these patients and their ventilators (excluding multiple isolations) were indistinguishable.

Table 3. *Phage-typing patterns of pyocine type S strains*

Strain	Phage-typing pattern
S^{I}	7/16/44/68/F8/109/119x/1214
S^{II}	7/44/68/F8/109/352/1214
S^{III}	7/21/31/F8/109/119x/352/Coll 11

DISCUSSION

The three groups into which these patients fall each illustrate something of the probable epidemiology of *Ps. aeruginosa* respiratory tract infection following tracheostomy and mechanical ventilation.

The first group comprised two patients who were already infected before tracheostomy was performed: both had chronic bronchitis and cor pulmonale. It seems likely that such patients as these were responsible for some of the initial contamination of our mechanical ventilators.

The second group was made up of four patients who did not become infected. It is striking that in each case contamination of the ventilator was never demonstrated, a situation found in only two instances in the ventilators of those patients who did acquire infection.

The third and most important group was made up of the 12 patients who did become infected. In 11 of the 12, simultaneous sampling of tracheo-bronchial secretions and of the ventilators was carried out repeatedly until infection was established. In seven cases contamination of the ventilator was demonstrated before respiratory tract infection; in two infection developed in the absence of demonstrable contamination; and in two more infection was detected before contamination.

In the interpretation of these findings, it should be noted that the method of sampling was relatively inefficient, as in the interest of practicability it was possible only to sample four sites in the ventilator, chosen on the basis of a previously demonstrated high likelihood of contamination when other parts were contaminated. Thus detection of contamination depended on widespread colonization of the moist parts of the machine from limited foci not touched by our standard regime of disinfection. In contrast, sampling of the patients' tracheo-bronchial secretions was presumed to be more sensitive. The failure to detect contamination of the ventilator with *Ps. aeruginosa* before colonization of the patient's respiratory

tract in four instances is therefore not surprising, and the success in seven cases the more striking.

The results of pyocine and phage typing lend further support to the hypothesis of machine-to-patient spread. The finding of 15 indistinguishable strains out of 22 isolated—and these of an unusual pattern—is in striking contrast to the general distribution of pyocine typing patterns. Among strains of *Ps. aeruginosa* isolated from the respiratory tracts of 52 other patients in hospital during the same time, only two were of type S, the majority being types A, B or D, a distribution similar to that described by Darrell & Wahba (1964). Self-infection, in view of the predominance of a single pyocine type and the demonstration of faecal carriage in only one patient, seems unlikely. The most convincing explanation is cross-infection, and the only evident common source the ventilators. Patients nursed in distant wards acquired the same organism, which was otherwise only demonstrated in their immediate vicinity and in the ventilators, and never in the general environment of the hospital. Even where patients were nursed in the same ward they were usually separated in time, so that other infected patients are not a likely direct source.

In explanation of the importance of these findings, some discussion of the meaning of the word 'infection' in this context is relevant. Often the presence of *Ps. aeruginosa* in the respiratory tract is of little clinical importance. However, eight of these patients died, and in six of the seven cases where an autopsy was performed there was evidence of bronchopneumonia. In only three was bacteriological study possible: in two of these *Ps. aeruginosa* was the only organism isolated from areas of consolidation, while in the third *Ps. aeruginosa* was confined to the trachea and main bronchi and *Staph. aureus* was isolated in pure culture from the areas of bronchopneumonia, although it had not been found in the endotracheal aspirate. These cases leave no doubt that the finding of *Ps. aeruginosa* in the sputum may be an indication of true infection. It follows that patients treated by mechanical ventilation must be protected against acquiring infection from the ventilators, either by the use of ventilator components which are readily sterilizable or disposable, or by the use of filters to isolate the patient from the machine.

SUMMARY

Eighteen patients treated by tracheostomy and mechanical ventilation were studied in an attempt to determine whether contaminated ventilators could act as a source of *Pseudomonas aeruginosa* isolated from the respiratory tract. Twelve became infected. In seven instances it was possible to demonstrate prior contamination of the ventilator. Furthermore, eight of the patients had indistinguishable organisms on the basis of pyocine and phage typing, and six of the ventilators harboured the same organisms. The most likely explanation is cross-infection via contaminated machines.

I thank members of the consultant staff of St Thomas's Hospital for permission to study these patients and for helpful discussion, Sister S. Smith for collecting many of the specimens, Miss Susan Madden for careful technical help, Mrs E. H. Asheshov for phage typing and Dr M. Ridley and Dr M. T. Parker for continued good advice.

REFERENCES

Alder, V. G., Brown, A. M. & Gillespie, W. A. (1966). Disinfection of heat-sensitive material by low-temperature steam and formaldehyde. *J. clin. Path.* **19**, 83.

Bishop, C. (1966). *Pseudomonas aeruginosa* cross-infection. *Lancet* i, 267.

Bishop, C., Potts, M. W. & Molloy, P. J. (1962). A method of sterilization for the Barnet respirator. *Br. J. Anaesth.* **34**, 121.

Bishop, C., Roper, W. A. G. & Williams, S. R. (1963). The use of an absolute filter to sterilize the inspiratory air during intermittent positive pressure respiration. *Br. J. Anaesth.* **35**, 32.

Darrell, J. H. & Wahba, A. H. (1964). Pyocine typing of hospital strains of *Pseudomonas pyocyanea*. *J. clin. Path.* **17**, 236.

Gaby, W. L. & Hadley, C. (1957). Practical laboratory test for the identification of *Pseudomonas aeruginosa*. *J. Bact.* **74**, 356.

Haynes, W. C. (1951). *Pseudomonas aeruginosa*—its characterization and identification. *J. gen. Microbiol.* **5**, 939.

Haynes, W. C. & Rhodes, L. J. (1962). Comparative taxonomy of crystallogenic strains of *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis*. *J. Bact.* **84**, 1080.

Hugh, R. & Leifson, E. (1955). The taxonomic significance of fermentative versus oxidative metabolism of various Gram-negative bacteria. *J. Bact.* **66**, 24.

Phillips, I. & Spencer, G. (1965). *Pseudomonas aeruginosa* cross-infection, due to contaminated respiratory apparatus. *Lancet* ii, 1325.

Sykes, M. K. (1964). Sterilizing mechanical ventilators. *Br. med. J.* i, 561.

Thornley, M. J. (1960). The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *J. appl. Bact.* **23**, 37.

Wahba, A. H. (1964). Epidemiology of hospital infection with *Pseudomonas pyocyanea*. Thesis, University of London.

J. Hyg., Camb. (1967), **65**, 237
Printed in Great Britain

The isolation of salmonellas from animal feedingstuffs

BY R. W. S. HARVEY AND T. H. PRICE
Public Health Laboratory, Cardiff

(*Received* 6 *January* 1967)

In a previous publication (Harvey, Mahabir & Price, 1966) a motility technique was described for the isolation of salmonellas by secondary enrichment. The method was independent of selectively toxic chemicals and was applicable to the culture of a wide range of serotypes. The method was not original as it had been used by Gilbert & Fournier (quoted Carnot & Garnier, 1902) and by Carnot & Garnier (1902) as a primary enrichment process. By primary enrichment we mean culture of the original infected material in an enrichment medium (fluid or semi-solid). Secondary enrichment consists of further enrichment of an inoculum derived from the incubated primary enrichment broth. The use of the Craigie tube (1931) by Jones & Handley (1945) is an example of a differential motility technique used for secondary enrichment and the employment of a broth filled U-tube with arms divided by a sintered glass filter (Pijper 1952) illustrates the method used for primary enrichment. Recently a similar method has been described by Stuart & Pivnick (1965). In this case the motility technique was combined with the use of selectively toxic chemicals and the method used for primary enrichment.

The use of capillary pipettes filled with 0·15–0·2 % nutrient agar in which agglutinating serum was incorporated had proved an invaluable method for isolating single salmonella serotypes from a mixture in an orderly and predetermined manner (Harvey & Price, 1962). In the later stages of this investigation (unpublished) we discovered that the migration of a mixture of salmonellas and other organisms through a column of 0·15 % nutrient agar (without agglutinating serum) increased the relative proportion of salmonellas to other organisms appearing at the uninoculated surface of the agar. This observation aroused our interest and the technique became a profitable routine part of our examination of crushed bone for salmonellas. It was natural to extend the use of the method to the examination of abattoir swabs and polluted water samples and the results of this investigation were recently published (Harvey *et al.* 1966). The arrival in the laboratory of regular consignments of animal feedingstuffs infected with salmonellas suggested that the differential motility technique might be used for their examination and a trial of methods was, therefore, inaugurated.

MATERIALS AND METHODS

The materials examined were samples of commercial meat and bone meal, 250–300 g. in weight. All were produced by one firm. The firm's suppliers indicated that, as far as they were aware, the raw materials were of British origin.

Carnot & Garnier (1902) had found the apparatus used by Gilbert & Fournier

to be too complicated and too fragile for routine purposes. In our turn, we considered that the capillary pipettes, which had proved so useful in the Indian bone examination and in the secondary enrichment of salmonellas in abattoir samples and polluted water, to be lacking in strength for our routine practice. We, therefore, decided to modify the Craigie tube (Craigie, 1931), so that it could be used for our differential motility technique. A screw-capped 1 oz. (approximately 28 ml.) universal container was used. Inside this was placed a tube of dimensions 7 × 1 cm., open at both ends. Within this tube there was a glass rod 3·5 cm. in length with expanded ends, its distal end resting on the bottom inside surface of the universal container as in Fig. 1. The interior of the container was filled with 0·15 % nutrient agar to a depth of 5 cm. This concentration was chosen for convenience as it was obtained by diluting ordinary nutrient agar 1 in 10 with nutrient broth.

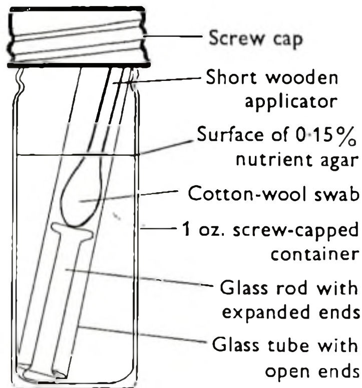


Fig. 1. Modified Craigie tube.

The technique used in this investigation was to culture four 25 g. portions of each separate specimen of feedingstuff. Each 25 g. sample was placed in an 8 oz. (approximately 220 ml.) wide-mouthed screw-capped jar and covered with 75 ml. of nutrient broth. The whole was incubated for 24 hr. at 37° C. After culture in broth, 75 ml. of double strength selenite F broth was added and the container and contents transferred to a water bath and incubated at 43° C. In this laboratory we have used this temperature for many years for the isolation of salmonellas from heavily contaminated samples such as sewage and abattoir swabs (Harvey & Thomson, 1953). This temperature is essential in our area for the examination of abattoir specimens, as without its use a statistically significant number of positive samples will be falsely reported as negative (unpublished observations). Subcultures were made from the selenite F broths at 24, 48 and 72 hr. on de Loureiro's (1942) modification of Wilson and Blair's medium. These plates were incubated for 48 hr. at 37° C. and suspicious colonies were picked and inoculated on the slope and in the condensation water of small agar slopes in bijou bottles for further investigation. These, after 6 hr. incubation at 37° C., showed sufficient turbidity in the water of condensation for satisfactory slide agglutination to be performed

with H agglutinating antiserum. Thus both the O antigen and one of the H antigens could frequently be identified 6 hr. after picking the colony. Biochemical reactions were seldom employed and complex screening media were rarely used. When the Wilson and Blair plates (inoculated from selenite broth after 24 hr. incubation) had been examined for salmonella colonies, they were subjected to secondary enrichment. This procedure was carried out on all such plates, whether negative or positive by the primary examination. The plates inoculated from selenite broth at 48 and 72 hr. were not subjected to secondary enrichment. The enrichment was carried out in the following manner: sterile cotton-wool swabs on wooden applicators 5 cm. long were rubbed over the surface of each plate to remove the surface growth. The charged swab was then introduced into the upper end of the 7 × 1 cm. tube inside the universal container (Fig. 1), with the cotton-wool resting on the upper end of the glass rod. The culture-coated swab was, therefore, covered with 0·15 % nutrient agar. The inoculated modified Craigie tube was incubated at 37° C. for 24 hr. during which time growth appeared on the agar surface outside the 7 × 1 cm. tube. The growth was subcultured to brilliant green MacConkey agar (Harvey, 1956), and the plates were incubated at 37° C. for 24 hr. and examined for suspicious colonies in the usual way.

RESULTS

As this paper is intended to illustrate the use of a method of secondary enrichment for salmonella isolation in routine laboratory practice, it is best to consider the results obtained by primary and secondary enrichment separately.

Table 1. *Number of positive specimens obtained after subculture at various times*

	Subculture from selenite broth after incubation at 43° C. for		
	24 hr.	48 hr.	72 hr.
Positive plates	46	19* + 6†	18* + 5†
Total positive samples	46	52	57

* Plates from samples positive on previous subculture(s).
† Plates from samples not positive before.

Primary enrichment

It is evident (Table 1) that the majority of specimens are positive at the 24 hr. subculture from selenite F broth. Until the development of secondary enrichment, multiple subculture was regarded as being essential to efficient salmonella isolation. It has been used routinely in this laboratory since we first studied the survival of *S. paratyphi* B in sewers (Harvey & Phillips, 1955). In this technique it is not only necessary to consider the number of positives obtained at single subculture times, but also to observe the cumulative total positives found at 24 hr., 24 + 48 hr. and 24 + 48 + 72 hr. These results are also given in Table 1.

In this series, if the examination had ceased with the 24 hr. subculture, 11 positive samples would have been reported as negative.

Secondary enrichment

The results of secondary enrichment are given in Table 2.

In this series, four samples would have been reported as negative had the primary enrichment plates not been examined. All these four samples were negative at the 24 hr. subculture, but were positive either at 48 hr., or 72 hr., or at both times. All plates which were positive on the primary Wilson and Blair plates subcultured from selenite at 24 hr. were also positive when subjected to the secondary enrichment technique. If secondary enrichment alone had been relied on for the routine examination, only 3 % of the total possible positives would have been missed. These findings were important to the development of the routine examination that was ultimately adopted.

Table 2. *Secondary enrichment results*

Size of sample	25 g.
Total no. of samples	816
Total samples positive by primary enrichment alone	57 (42)
Total samples positive by secondary enrichment alone	133 (97)
Total samples positive by primary and secondary enrichment combined	137 (100)

Figures in parentheses show success rate as percentage of total positives obtained by primary and secondary enrichment combined.

Table 3. *Effect of sample size on the number of positives obtained*

Primary and secondary enrichment combined		Size of total sample
No. of positives obtained from 1 × 25 g. samples	30 (15)	25 g.
No. of positives obtained from 2 × 25 g. samples	43 (21)	50 g.
No. of positives obtained from 3 × 25 g. samples	54 (26)	75 g.
No. of positives obtained from 4 × 25 g. samples	62 (30)	100 g.
Total no. of 100 g. samples		204

Figures in parentheses show success rate as a percentage of the total number of 100 g. samples.

Effect of sample size on percentage success

It will be noted that in Tables 1 and 2, the sample size was 25 g. The effect of increasing the size of sample in stages up to 100 g. was also investigated. Four samples of 25 g. from each specimen of animal feed submitted by the manufacturer were separately examined. The first 25 g. was labelled A, the second B, the third C and the fourth D. The results are given in Table 3.

Table 3 shows that a fourfold increase in sample size doubled the percentage of positive results.

Serotypes isolated from animal feedingstuffs

As the infrequency of isolation of certain common serotypes from animal feedingstuffs is often quoted as evidence for their minor importance in the epidemiology of salmonellosis, it is worth while recording the serotypes encountered. In this connexion, it has been our experience that a detailed examination of a few specimens of the raw materials used as constituents for animal feed gives more useful information than the cursory examination of many samples. The cleavage of opinion as to the best approach to salmonella isolation techniques has been previously commented on by Felsenfeld (1945), though he did not commit himself as to preference. We record in Table 4 the information obtained separately by primary and secondary enrichment on the salmonella serotype content of the animal feedingstuff samples examined.

Table 4 records that *S. dublin* was not isolated and that *S. typhi-murium* was isolated three times. Eleven serotypes were isolated by secondary enrichment not found by primary enrichment. Two serotypes were found by primary enrichment only.

Table 4. *Salmonella serotypes isolated from* 100 *g. samples*

Serotype	No. of isolations by primary enrichment	No. of isolations by secondary enrichment
S. adelaide	0	1
S. alachua	1	0
S. anatum	0	3
S. binza	0	2
S. bonariensis	0	1
S. bredeney	0	2
S. california	5	6
S. cerro	1	1
S. cubana	0	3
S. derby	1	2
S. eimsbuettel	5	9
S. glostrup	1	1
S. havana	0	1
S. infantis	1	2
S. johannesburg	1	2
S. kentucky	0	1
S. kiambu	0	1
S. lexington	0	1
S. livingstone	0	2
S. meleagridis	1	2
S. mikawasima	1	2
S. minnesota	1	0
S. newington	3	3
S. oranienburg	0	2
S. senftenberg	7	7
S. singapore	0	2
S. tennessee	4	9
S. thomasville	1	2
S. typhi-murium	1	3
S. unidentified	0	1
No. of identified serotypes	16	27

DISCUSSION

It will be noted that we have used preliminary culture of meat and bone meal samples in nutrient broth before culture in the fluid enrichment medium. This accords with the practice of Jepson (1957) and with our own experience of the isolation of salmonellas from dried material such as crushed bone. Not all salmonella serotypes are more easily recovered by incubation in broth before enrichment. *Salmonella typhi* in naturally polluted water is better isolated by immediate enrichment in selenite F broth. The isolation of *S. typhi* is, however, a separate problem.

The success of secondary enrichment is unlikely to be due to any inefficiency of the primary plating medium for the reasons given in our previous paper (Harvey *et al.* 1966). Whether Wilson and Blair, deoxycholate, S.S. agar or brilliant green MacConkey agar is used as primary plating medium, there is no doubt that the selective motility method of secondary enrichment greatly improves the results.

The best combination of primary plating medium (the selective agar used for subculture from selenite F broth) and secondary plating medium (the selective plating medium for subculture from the agar surface of the Craigie tube), has not yet been evaluated. The amount of labour involved in such complex trials is very great as large numbers of specimens and plates have to be examined. Wilson and Blair or brilliant green MacConkey are, however, eminently suitable for secondary plating media, and we have a strong preference for the latter as it is the only selective agar that adequately suppresses proteus species and it requires only 18–24 hr. incubation. It is true that positive results can be obtained with deoxycholate citrate agar used for secondary plating, but more labour is involved in picking colonies owing to the ease with which proteus species grow on this medium. Differentiation between salmonella and proteus colonies on deoxycholate plates can undoubtedly be improved by incubating them for 48 hr. The Wilson and Blair and brilliant green MacConkey agars plated from the Craigie tubes, in contrast to the deoxycholate plates, can be separated into negatives and presumptive positives on sight and suspicious colonies checked by slide agglutination. In some cases, the brilliant green MacConkey plates used for secondary plating from the Craigie tubes are almost pure cultures of salmonellas. Occasionally citrobacter strains on brilliant green MacConkey can be confused with salmonellas. This, however, rarely causes difficulty as these organisms can be readily distinguished from salmonellas by biochemical tests.

In the present investigation we observed the results obtained using Wilson and Blair as primary plating medium and brilliant green MacConkey as secondary plating medium. This combination was purposely chosen as, in our experience, differentiation of salmonella colonies is good on both these two media. Good differentiation is correlated with rapid diagnosis as no complex screening is necessary, apart from slide agglutination and ultimate confirmation of picked colonies by a salmonella reference laboratory. Rapid diagnosis is important as results are telephoned to the firm supplying the animal feedingstuffs as soon as positive O and

H slide agglutination is obtained with polyvalent sera. No errors have been made in the two years we have used the above technique.

The secondary enrichment technique was as quick as, and considerably more efficient than, the primary enrichment process. The method represents an attempt to employ a sensitive and selective technique (the two adjectives are not synonymous) in routine laboratory work. If a particular aspect of salmonella contamination of animal feedingstuffs required investigation such as the frequency of occurrence of *S. typhi-murium*, a more academic approach would be needed. For such research we think that the serological technique used in examining Indian crushed bone would be necessary (Harvey & Price 1962). By using a polyvalent H specific and non-specific serum from which the H phase I agglutinins of *S. typhi-murium* had been absorbed, the chances of recovery of the serotype might be improved and a truer estimate of its frequency obtained. A separate investigation is planned along these lines. In Indian material, the use of a progressive serological isolation process showed *S. typhi-murium* to be the sixth most common serotype encountered and the phage-types isolated often corresponded to those found in animals and man in the United Kingdom. We are not attempting to implicate Indian bones in the initiation of salmonellosis in this country. All inquiries indicate that manufacturers are afraid of using such material owing to the dangers of anthrax infection (Davies & Harvey, 1953, 1955; Jamieson & Green, 1955; Harvey, 1958). Examination of Table 4 raises some important questions. First, if the raw material is exclusively of British origin, why is *S. typhi-murium* not more evident? Why is the serotype distribution so exotic? Is there perhaps an admixture of imported material in the final product, or does it reflect plant contamination? The lack of isolation of *S. dublin* causes little surprise as, in our experience over a period of 10 years, *S. dublin* is poorly isolated on Wilson and Blair's medium. This applies both to our own laboratory prepared medium and to the main commercial brand of Wilson and Blair obtainable in this country. As we have not tested other formulae of Wilson and Blair we would hesitate to be sweeping in the interpretation of this phenomenon. Nevertheless, it should be taken into account in assessing the accuracy of figures showing the incidence of *S. dublin* infection.

Lastly, the dependence of successful isolation on the size of sample is well illustrated by Table 3. This is no new phenomenon, but was shown to be important in the isolation of salmonellas from American spray dried egg (Report, 1947), and more recently in the examination of animal feedingstuffs by Dutch workers (Jacobs, Guinée, Kampelmacher & van Keulen, 1963). The latter authors calculated that 10% of the bags of fishmeal imported into the Netherlands are probably contaminated with *S. typhi-murium*.

SUMMARY

A technique is described for the routine isolation of salmonellas from meat and bone meal samples used in animal feedingstuffs. The technique more than doubled the number of positives obtained by an orthodox method. Identical samples were used in the trial.

We should like to thank Prof. Scott Thomson for his advice in the preparation of this paper, Dr G. J. G. King of the Public Health Laboratory, Bournemouth, for identifying the serotypes isolated, and Mr T. R. Liddington and Miss L. Williams for their technical assistance.

REFERENCES

Carnot, P. & Garnier, M. (1902). Sur la technique des cultures en tubes de sable. *C. r. Séanc. Soc. Biol.* **54**, 748.

Craigie, J. (1931). Studies on serological reactions of flagella of *B. typhosus*. *J. Immun.* **21**, 417.

Davies, D. G. & Harvey, R. W. S. (1953). Dried bones as a source of anthrax. *Lancet* ii, 880.

Davies, D. G. & Harvey, R. W. S. (1955). The isolation of *Bacillus anthracis* from bones. *Lancet* ii, 86.

Felsenfeld, O. (1945). The salmonella problem. *Am. J. clin. Path.* **15**, 584.

Harvey, R. W. S. (1956). Choice of a selective medium for the routine isolation of members of the salmonella group. *Mon. Bull. Minist. Hlth* **15**, 118.

Harvey, R. W. S. (1958). Anthrax from bone meal. *Br. med. J.* ii, 1040.

Harvey, R. W. S., Mahabir, D. E. & Price, T. H. (1966). A method of secondary enrichment for salmonellas independent of selectively toxic chemicals. *J. Hyg., Camb.* **64**, 361.

Harvey, R. W. S. & Phillips, W. P. (1955). Survival of *Salmonella paratyphi B* in sewers: its significance in investigation of paratyphoid outbreaks. *Lancet* ii, 137.

Harvey, R. W. S. & Price, T. H. (1962). Salmonella serotypes and arizona paracolons isolated from Indian crushed bone. *Mon. Bull. Minist. Hlth* **21**, 54.

Harvey, R. W. S. & Thomson, S. (1953). Optimum temperature of incubation for isolation of salmonellae. *Mon. Bull. Minist. Hlth* **12**, 149.

Jacobs, J., Guinée, P. A. M., Kampelmacher, E. H. & Keulen, A. van (1963). Studies on the incidence of salmonella in imported fish meal. *Zentbl. VetMed.* **10**, 542.

Jamieson, W. M. & Green, D. M. (1955). Anthrax and bone-meal fertilizer. *Lancet* i, 560.

Jepson, A. (1957). Bacteriological examination of manufactured meat products. *Monograph Ser. W.H.O.* no. 33, p. 420.

Jones, R. E. & Handley, W. R. C. (1945). A selective medium for the isolation of salmonella bacilli from heavily contaminated material. *Mon. Bull. Minist. Hlth* **4**, 107.

Loureiro, J. A. de (1942). A modification of Wilson and Blair's bismuth sulphite agar (stabilized stock solution). *J. Hyg., Camb.* **42**, 224.

Pijper, A. (1952). Dimensional differentiation, filtration and separation of bacteria. *J. Path. Bact.* **64**, 529.

Report (1947). The bacteriology of spray-dried egg with particular reference to food poisoning. *Spec. Rep. Ser. med. Res. Coun.* no. 360, p. 8.

Stuart, P. F. & Pivnick, H. (1965). Isolation of salmonellae by selective motility systems. *Appl. Microbiol.* **13**, 365.

J. Hyg., Camb. (1967), 65, 245
Printed in Great Britain

Reactions and antibody response to live influenza vaccine prepared from the Iksha (A2) strain. (A report to the Medical Research Council by their Committee on Influenza and other Respiratory Virus Vaccines*)

BY A. S. BEARE AND D. A. J. TYRRELL
Common Cold Research Unit, Salisbury

J. C. McDONALD,† T. M. POLLOCK AND C. E. D. TAYLOR
Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

C. H. L. HOWELLS
New Cross Hospital, Wolverhampton

AND L. E. TYLER
Wednesfield, Wolverhampton

(*Received* 18 *January* 1967)

INTRODUCTION

Live influenza vaccine has been used extensively in the U.S.S.R. (Ritova, Zhukovsky & Yevstigneyeva, 1963; Smorodintsev, Alexandrova, Chalkina & Selivanov, 1965), in eastern Europe (Starke, Siebelist & Hiller, 1964) and occasionally in other countries (Okuno & Nakamura, 1966). In England five trials of this vaccine have been made since 1960; the first two during 1960–61 (McDonald, Zuckerman, Beare & Tyrrell, 1962) and the third in 1962 (Andrews *et al.* 1966).

The findings from these three trials were encouraging in that serological evidence of symptomless infection was produced by the vaccine in most recipients and the volunteers were apparently resistant to re-infection with a challenging dose of live virus given intranasally 1 month later.

Accordingly, in 1963 a field trial was made to try to assess the protection conferred against influenza. Approximately 1500 personnel of the Royal Air Force were vaccinated and a similar number who acted as controls received an inert fluid.

The antibody response to the batch of vaccine used in this trial was much less satisfactory than in the previous investigations, and did not suggest that adequate protection had been induced. However, there was no outbreak of influenza during the follow up, and although a few cases of influenza were observed in both the

* Members of Committee: Prof. C. H. Stuart-Harris (*Chairman*), Dr B. E. Andrews, Dr A. S. Beare, Prof. G. Belyavin, Prof. G. W. A. Dick, Prof. Sir Austin Bradford Hill, Mr I. D. Hill, Dr F. Himmelweit, Dr W. W. Holland, Dr J. W. Howie, Dr F. O. MacCallum, Dr H. G. Pereira, Dr F. T. Perkins, Dr A. T. Roden, Dr D. A. J. Tyrrell, Dr T. M. Pollock (*Secretary*).

† Present address: Department of Epidemiology and Health, McGill University, Montreal.

vaccinated and control groups their numbers were too small to permit valid conclusions about efficacy. The aspects of this trial concerned with the clinical evidence of protective efficacy have therefore not been reported. However, the antibody estimations which were made in some participants are described below.

A fifth trial designed to assess response and reactions in children and adults was made in 1965, since vaccine which was satisfactory for adults might nevertheless produce more severe reactions in children. This investigation provided an opportunity to check the results of the previous (1963) trial on a small scale. The findings from this trial in children and adults are also described here, and the vaccination reactions and antibody responses observed in all five trials are reviewed.

MATERIALS AND METHODS

Virus isolation

In the fourth trial, throat swabs were broken off into transport medium consisting of Hanks's balanced salt solution (BSS), containing 0·5 % lactalbumin hydrolysate (LAH) and 0·02 % $NaHCO_3$; this was replaced in the fifth trial by BSS and 2 % bovine plasma albumin (BPA). Specimens were taken to the laboratory in thermos flasks containing water ice. In trial 4, virus isolation was attempted by amniotic inoculation in five 10-day embryonated eggs followed by a blind passage in three eggs; in trial 5, the culture system consisted of two tubes of monkey kidney tissue culture which were tested for the presence of a haemadsorbing agent after 5 days incubation in a rolling drum at 33° C.

Antibody titrations

Haemagglutination-inhibiting (HI) antibodies were titrated by methods described by the WHO Expert Committee on Respiratory Virus Diseases (1959) and by Beare (1962). Receptor Destroying Enzyme (RDE) was obtained from Burroughs Wellcome and Company. All tests were performed in WHO plastic plates. Viruses used in the HI tests were: (1) a strain of A2/Singapore/1/57 which had received many egg passages, (2) a fifth egg passage of the 1964 A2 virus known as A2/England/12/64, and (3) a strain called A2/England/7/65 which had had an unspecified number of egg passes. The viruses were diluted in saline to contain eight 50 % haemagglutinating doses per 0·25 ml. volume.

Complement-fixation tests were performed with soluble A antigen by overnight fixation at 4° C.

The technique of the neutralization test has been described (Pereira, 1958; Beare, 1962). In the fifth trial, neutralization tests and preliminary virus titrations were done at 33° C. instead of the 35° C. temperature originally used. This was for convenience only and was considered unlikely to make any practical difference. A2/Singapore/1/57 was at all times the test virus.

TRIAL 4: IN ROYAL AIR FORCE

General plan

During November and December, 1963, 2950 apprentices or boy entrants aged 15–18 years received live vaccine or control fluid intranasally. Ninety-two from the 1468 given live vaccine were bled at the time of vaccination and again 14 days later. Throat swabs taken from 77 participants 48 and 72 hr. after vaccination were examined for the presence of the vaccine virus.

The vaccine

A sixth allantoic pass of the Russian A2 Iksha virus used in the previous trials was inoculated intranasally into a human volunteer. Virus recovered from throat washings was grown allantoically in leucosis-free eggs by Dr F. Himmelweit of the Wright-Fleming Institute of Microbiology.

The pool of vaccine was titrated by Dr H. G. Pereira of the World Influenza Centre who reported a haemagglutination titre of 640 and an infectivity titre of 10^9 50 % egg infective doses (EID50) per ml. The vaccine strain grew readily in embryonated eggs and was antigenically closely related to A2/Singapore/1/57; but, as before, it differed from the latter in that it was inhibitor resistant.

The vaccine pool was tested for safety by the procedures described by Andrews *et al.* (1966). Paired blood specimens from the volunteer from whom the vaccine virus was recovered were examined by Dr R. J. C. Harris for antibody against Resistance Inducing Factor (RIF) with negative results.

In October 1963, immediately before the trial began, two ampoules of vaccine selected at random were re-tested by Dr Pereira for virus content; one of these ampoules had a titre of $10^{9.1}$ EID50 per ml. and the other $10^{8.8}$ EID50 per ml. In using the vaccine for the trial the lower estimate was taken and a dilution of 1/50 was assumed to contain about 10^7 EID50/ml.

Methods of storage and dilution of vaccine were as previously described (Andrews *et al.* 1966).

Dose

Forty of the participants tested serologically received a dose of 10^6 EID50 of virus and the remainder a dose of 10^7 EID50. Each dose was contained in a volume of 0·5 ml., half of which was given into each nostril; after instillation of the vaccine, participants were asked to sniff gently and not to blow their nose for at least 15 min.

RESULTS

Virus isolation

No influenza virus was isolated from the 77 throat swabs examined.

Antibody response to vaccination in volunteers for special tests

As in the previous trials the results were considered according to whether the participants had or had not neutralizing antibody before vaccination.

The antibody response to vaccination after each dose of vaccine in recruits with and without neutralizing antibody is shown in Table 1. It is evident that this result is poor. The response was slightly better with the larger dose; but less than half of the participants without pre-vaccination neutralizing antibody produced an HI rise against the 1957 virus, while the proportion of those *with* prevaccination antibody who responded similarly is less than one-fifth. When A2/England/12/64 is used as the indicator strain, the result is equally disappointing.

In no group was there a significant production of antibody to soluble antigen.

Table 1. *Antibody response in subjects with and without pre-vaccination neutralizing antibody given different strengths of vaccine*

Pre-vaccination neutralizing antibody	Dose EID 50	Antibody response according to three tests: HI* A 2/Sing/1/57	HI* A 2/Eng/12/64	CF*
Absent	10^6	2/13	1/11	0/12
	10^7	8/21	4/19	2/20
Present	10^6	1/25	2/21	0/21
	10^7	5/28	8/28	0/27

* Fourfold or greater rise.

TRIAL 5: IN CHILDREN AND ADULTS

General plan

This trial was made in March 1965 among 31 children resident in Wednesfield Cottage Homes, Staffordshire, and in adult members of the staff. The children were placed in two groups according to age. Group 1 contained children aged from 11 to 15 years and group 2 those from 7 to 10 years. The adults were immunized on the first day of the trial, the group 1 children on the third day and the group 2 children on the fifth day. In this way it was possible to observe any reactions in older participants before giving the vaccine to the younger ones.

A nasal and a throat swab were taken from each participant before vaccination and examined in monkey kidney cell cultures for haemadsorbing agents. Forty-eight hours after vaccination a second throat swab was taken and an attempt made to isolate virus. Serum samples were withdrawn before vaccination and approximately 3 weeks later. Oral temperature readings were recorded before vaccination and each night and morning for the next forty-eight hours.

The vaccine

A new vaccine pool was prepared by propagating the virus of trial 4 in leucosis-free eggs supplied by the Poultry Research Station, Houghton Grange. This virus had now had six allantoic passages, one passage in humans and two further allantoic passages. In general, the virus was very similar to that of the previous vaccine: the haemagglutinating titre (reciprocal dilution) was 640 and the in-

fectivity titre $10^{8\cdot8}$ EID50 per ml. Identification tests by cross-haemagglutination-inhibition indicated that it was similar to the original and later Iksha vaccines except that the virus had undergone a slight increase of inhibitor-sensitivity. Safety tests for bacterial sterility and absence of other adventitious agents were uniformly negative.

Storage and transport of vaccine

The vaccine was stored at -70° C. and was kept in a mixture of solid carbon dioxide and alcohol during transport. At each vaccination session an ampoule was thawed immediately before use. Part of the contents was then diluted in Hanks's BSS plus 0·5 % gelatin adjusted to pH 7·2 and without antibiotics. Diluted and undiluted vaccine from the same ampoule was returned to Colindale on ice and inoculated into eggs.

Vaccination procedure

Each participant lay with the head hanging back over the side of a bed. Three drops (each 0·02 ml.) of vaccine diluted 1/7·6 (total dose 10^7 EID50) were then instilled into each nostril.

Participants remained in this position for 1 min. after vaccination and were instructed to sniff after getting up.

Testing of potency of vaccine used at Wednesfield

Samples of the actual vaccine dilutions used on days 1, 3 and 5 of the trial were titrated in eggs and indicated that the calculated number of EID50 of live vaccine had in fact been administered throughout.

Results

There were 16 adults and 31 children of whom 12 were aged 7–10 years and 19 aged 11–15 years. In three children, all in the group aged 7–10 years, a slight rise in temperature to 99° F. was found in the 24 hr. after vaccination. No constitutional reactions occurred in any participant.

No viruses were isolated in monkey kidney cells from swabs taken before or after vaccination either at Colindale or at Wolverhampton.

An attempt was made to isolate virus in eggs from post-vaccination swabs without success.

Antibody titres: before vaccination

The pre-vaccination antibody titres in children and adults in all the tests are shown in Table 2. It is clear that a substantial proportion of both adults and children possessed neutralizing, HI, and CF antibodies before vaccination. It is also evident that, apart from those demonstrated by complement-fixation, antibodies in children were found more frequently than in adults.

Antibody: response to vaccination

The antibody response to vaccination is shown in Table 3. The proportion responding with a fourfold rise of antibody was low both in those with and in those

without pre-vaccination antibody. There was no apparent difference in the response of children and adults.

The analysis shown in the table includes participants with all levels of antibody, and the participants with high pre-vaccination antibody might not be expected to show a fourfold response to vaccination. Thus, for those who responded with a fourfold or greater rise in neutralizing antibody the geometric mean titre before vaccination was 26·9, whereas the initial geometric mean titre of those who failed to respond was much greater—140·7. However, this was not so for HI antibody. The initial geometric mean titres HI antibody (A2/Singapore/57) were almost identical for those who did and did not respond—50·4 and 50·1 respectively.

Table 2. *Wednesfield Cottage Homes: antibody titres before vaccination in children and adults*

Type of antibody	Participants	Antibody present
Neutralizing A2/Sing/57	Adults	14/16
	Children	31/31
HI A2/Sing/57	Adults	10/16
	Children	30/31
HI A2/Eng/65	Adults	7/16
	Children	21/31
CFT	Adults	15/16
	Children	20/31

Table 3. *Fourfold or greater antibody rise to vaccination in participants with and without each type of antibody before vaccination*

	Post-vaccination antibody response			
Pre-vaccination antibody	Neutralizing A2/Sing/57	HI A2/Sing/57	HI A2/Eng/65	CFT
Absent	1/2	3/7	3/19	3/12
Present	7/45	3/40	0/28	1/35

DISCUSSION

The two trials of live influenza vaccine described above are the last of a series of five undertaken in England since 1960. Though the purpose and complexity of the five trials varied, observations on reaction and antibody response to the Iksha strain were common to them all. The findings of the trials showed some variation and the differences encountered have accordingly been discussed below.

Some relevant characteristics of each trial are shown in Table 4. There are basic similarities in all the trials; adults or adolescents were included in each, and in each at least some participants were given the same large dose of vaccine, 10^7 EID 50, by the same method—nasal drops. Three batches of vaccine were used: batch 1 for trials 1, 2 and 3; batch 2 for trial 4 and batch 3 for trial 5.

In each trial, with the exception of trial 2, serum antibodies in some participants were estimated before and after vaccination. The trials thus provide an indication

of the antibody response to each of the three batches of vaccine used. The trials also give some indication of the frequency and character of reactions produced by three batches of vaccine.

Table 4. *Trials of live influenza vaccine in England: participants, preparation and dose of vaccine*

Trial	Year	Participants	Preparation of vaccine	Dose EID 50	No. of doses
1	1960	Adolescents and adults	Batch 1. Six passes in eggs	10^6, 10^7	Usually 2
2	1961	Adults		10^7	1
3	1962	Adults		10^7	1
4	1963	Adults	Batch 2—batch 1 plus one pass in man and one further egg pass	10^6 10^7 10^7	1
5	1965	Adults, and children 7–15 years	Batch 3—batch 2 plus one further pass in eggs	10^7	1

Table 5. *Reactions to vaccination in five trials*

Trial	Neutralizing antibody present or not known before vaccination			Neutralizing antibody absent before vaccination		
	No. vaccinated	Febrile reactions	Afebrile reactions	No. vaccinated	Febrile reactions	Afebrile reactions
1. R.A.F. 1960–61 (A)	42	0	0	22	3	0
2. R.A.F. 1960–61 (B)	513 (513 controls)	6 (8)	33 (33)	—	—	—
3. R.A.F. 1962	53	0	5	66	0	30
4. R.A.F. 1964	1468	0	Not known			
5. Wednesfield 1965	45	0	0	2	0	0

Vaccination reactions

The only reaction encountered was coryza, occasionally with fever (Table 5). Afebrile coryzal symptoms were recorded most frequently in trial 3, mainly in volunteers without neutralizing antibody, but since there was no unvaccinated group available for comparison, reactions cannot necessarily all be attributed to the vaccine. There were no febrile reactions with batch 2 of vaccine used in trial 4 or reactions of any kind with batch 3 in children or adults in trial 5. These two batches also gave a poorer antibody response than batch 1.

Antibody response

In Table 6 a comparison has been made of the frequency of virus recovery and antibody response in the participants in each of the four trials who were given

10^7 EID50 and for whom pre- and post-vaccination serum samples were available. It is evident that only a small proportion of participants without pre-vaccination antibody responded. The proportion of participants *with* pre-vaccination antibody who responded to batch 2 and to batch 3 is also small. The better results with batch 1 suggest that the virus underwent some change during its passage in man and its subsequent recovery in leucosis-free eggs. This process may have selected a virus which was too attenuated to stimulate an antibody response in a partially immune subject. However, an *increase* of virulence following human passage would usually be expected.

Table 6. *HI antibody response (fourfold or greater rise) and virus recovery in each of four trials among participants with and without pre-vaccination neutralizing antibody*

	Trial no.	Trial	No. of doses	No. tested and responding	Virus isolated
With pre-vaccination neutralizing antibody	1	R.A.F. 1960	2	24/42 (57 %)	5/42
	3	R.A.F. 1962	1	17/27 (63 %)	1/27
	4	R.A.F. 1963	1	5/28 (18 %)	0/28
	5	Wednesfield	1	5/45 (11 %)	0/45
Without pre-vaccination neutralizing antibody	1	R.A.F. 1960	2	4/22 (18 %)	12/22
	3	R.A.F. 1962	1	5/29 (17 %)	10/31
	4	R.A.F. 1963	1	8/21 (38 %)	0/21
	5	Wednesfield	1	1/2	0/2

It is also possible that variations in response were occasioned by changes in the immunity of the population since the start of the Asian epidemic. However, Hobson, Gould & Flockton (1967) found certain differences between the laboratory characteristics of the virus contained in batch 1 and of that contained in batches 2 and 3; and it seems likely that the infectivity or antigenicity of the Iksha strain has become modified by successive passage.

The better response to batch 1 in participants *with* pre-vaccination antibody suggests that a response may be more likely in the presence of previous sensitization by a related antigen. Persons without pre-vaccination antibody may, therefore, require several doses of live influenza vaccine for effective immunization, and it is now the Russian practice to use three doses of vaccine. Multiple inoculations may also be an advantage because of the possession by some volunteers of a temporary resistance induced by natural infection with other viruses such as rhinoviruses (Cate, Couch & Johnson, 1964).

Finally, as a result of these trials, it appears that the Iksha strain of influenza A2 is not now suitable for general use. The strain was initially reasonably satisfactory but it has probably changed its properties with passage in man and eggs. In future work, attempts should be made to avoid shifts in the genetic constitution of otherwise suitable viruses by the use of such techniques as limit dilution passages. There is also a need to extend the studies such as those of Soloviev, Orlova, Porubel & Vasilieva (1961), Kolchurina (1966) and Hobson *et al.* (1967) to enable a selection to be made of immunogenic and non-virulent viruses on the basis of laboratory

studies. For this reason new strains of influenza A2 and B are being studied in man and in the laboratory and will be given limited trials in small groups of antibody-free adults and children when indicated.

SUMMARY

Two field trials of A2 live influenza vaccine (Iksha) are described; the clinical reactions and antibody response in three earlier trials are compared.

The antibody response in the last two trials was unsatisfactory and was inferior to that observed in the earlier investigations. This reduction in response was probably due to an alteration in the virus during further passage.

In trial 4 we are greatly indebted to Technical Training Command, Royal Air Force (P.M.O. Air Vice-Marshal T. C. Macdonald) for granting facilities for this trial. Much of the work fell on the medical officers in the six Stations where the trial was conducted and upon the staff of their departments; special thanks are due to Group-Captain H. W. Whittingham, Wing-Commanders J. A. Cooney and G. Gilchrist, and Squadron-Leaders A. R. Bone, J. D. MacAllister, J. G. Robertson and K. E. A. Underwood-Ground.

The trial was supervised by Dr R. V. Peters, and Dr A. J. Zuckerman assisted in the vaccination of the volunteers. The virological tests were performed at the Central Public Health Laboratory, Colindale; valuable assistance in this work was given by Mrs J. Coetzee.

In trial 5 we are greatly indebted to the Management Committee, to Mr F. Macho, Superintendent, and to the staff of the Cottage Homes. Our thanks are also due to Pfizer Ltd. who provided facilities for the preparation and testing of the vaccine. The trial was greatly assisted by Mr David Smith who undertook the dilution of the vaccine at the clinic and the antibody tests at Colindale.

REFERENCES

Andrews, B. E., Beare, A. S., McDonald, J. C., Zuckerman, A. J. & Tyrrell, D. A. J. (1966). Further trials of live influenza vaccine. *Br. med. J.* i, 637.

Beare, A. S. (1962). Neutralization and haemagglutination-inhibition tests in a live influenza vaccine trial. *Mon. Bull. Minist. Hlth* **21**, 167.

Cate, T. R., Couch, R. B. & Johnson, K. M. (1964). Studies with rhinoviruses in volunteers: production of illness, effect of naturally acquired antibody, and demonstration of a protective effect not associated with serum antibody. *J. clin. Invest.* **43**, 56.

Hobson, D., Gould, E. A. & Flockton, Hilary, I. (1967). Laboratory studies on a strain of Asian influenza virus used as a living vaccine. *J. Hyg., Camb.* **65**, 255.

Kolchurina, A. A. (1966). The study of genetic characteristics of influenza virus strains in connection with their pathogenicity. *Abstr. Pap. IX int. Congr. Microbiol., Moscow*, p. 385.

McDonald, J. C., Zuckerman, A. J., Beare, A. S. & Tyrrell, D. A. J. (1962). Trials of live influenza vaccine in the Royal Air Force. *Br. med. J.* i, 1036.

Okuno, Y. & Nakamura, K. (1966). Prophylactic effectiveness of live influenza vaccine in 1965. *Biken's J.* **9**, 89.

Pereira, M. S. (1958). Typing of *Q*-phase influenza-A virus by serum-neutralisation tests in monkey-kidney cultures. *Lancet* ii, 668.

Ritova, V. V., Zhukovsky, A. M. & Yevstigneyeva, N. A. (1963). Comparative study of the immunological properties of live influenza vaccine in volunteers. *J. Hyg. Epidem. Microbiol. Immun.* **7**, 272.

Smorodintsev, A. A., Alexandrova, G. A., Chalkina, O. M. & Selivanov, A. A. (1965). Experiences in the development of live vaccines against influenza and influenza-like respiratory infections. *Appl. Virol.: I annu. Symp., Boca Raton, Fla.*, 1964, p. 142.

Soloviev, V. D., Orlova, T. G., Porubel, L. A. & Vasilieva, I. N. (1961). Study of the genetic characters of vaccine strains of type A-2 influenza virus. *Probl. Virol.* **6**, 743.

Starke, G., Siebelist, I. & Hiller, E. (1964). Untersuchungen zur Frage der Anwendung von Influenza-Lebendimpfstoffen. I. Ergebnisse einer Prüfung von lebenden Influenza-A_2-Impfstoffen in einem kontrollierten Versuch an Freiwilligen. *J. Hyg. Epidem. Microbiol. Immun.* **8**, 37.

World Health Organization Expert Committee on Respiratory Virus Diseases (1959). First report. *Tech. Rep. Ser. Wld Hlth Org.* no. 170.

J. Hyg., Camb. (1967), 65, 255
Printed in Great Britain

Laboratory studies on a strain of Asian influenza virus used as a living vaccine. (A report to the Medical Research Council Committee on Influenza and other Respiratory Virus Vaccines*)

BY D. HOBSON, E. A. GOULD AND HILARY I. FLOCKTON
The Department of Bacteriology, University of Liverpool

(*Received* 18 *January* 1967)

Several clinical trials of living influenza virus vaccine prepared from the Russian A 2 strain Iksha have been conducted in this country since 1960 under the auspices of the M.R.C. Committee on Influenza and other Respiratory Virus Vaccines. In the initial trials (McDonald, Zuckermann, Beare & Tyrrell, 1962) the vaccine pool represented the 6th allantoic passage (pass L_6) of the virus. Most subjects given intranasal drops of this material developed symptomless infection with slight or moderate rises in haemagglutination-inhibiting (HI) antibody titres, and were resistant to subsequent experimental reinfection. However, in a further trial of Iksha vaccine prepared in exactly the same way (Andrews, Beare, McDonald & Zuckermann, 1966) virus was recovered from only a few inoculated volunteers and only one-third of the group (mainly those with pre-existing HI antibody) showed a significant serological response. In subsequent trials (Beare *et al.* 1967) the vaccine pools were prepared somewhat differently; Iksha virus which had been recovered in the throat washings of a volunteer inoculated with pass L_6 material was given one further allantoic pass in eggs from a flock of hens known to be free from infection with avian leucosis agents. Adult volunteers given this vaccine pool (pass $L_6Hu_1L_1$) showed no evidence of established experimental infection, and few developed significant rises in HI antibody. A vaccine pool of the virus after one further allantoic passage (pass $L_6Hu_1L_2$) was administered to children, and this also seemed ineffective.

No obvious differences in the composition of the volunteer groups or in the method of vaccine administration emerged to account for these discrepant results, and it seemed probable that the virus strain itself might have undergone some change in character during the production of the different vaccine pools. There is thus an obvious need to define laboratory criteria which would allow direct recognition of influenza virus pools which are suitable for use as living vaccine from those which are not. Accordingly, at the request of the MRC Committee on Influenza and other Respiratory Virus Vaccines, it was decided to compare the

* Members of Committee: Prof. C. H. Stuart-Harris (*Chairman*), Dr B. E. Andrews, Dr A. S. Beare, Prof. G. Belyavin, Prof. G. W. A. Dick, Prof. Sir Austin Bradford Hill, Mr I. D. Hill, Dr F. Himmelweit, Dr W. W. Holland, Dr J. W. Howie, Dr F. O. MacCallum, Dr H. G. Pereira, Dr F. T. Perkins, Dr A. T. Roden, Dr D. A. J. Tyrrell, Dr T. M. Pollock (*Secretary*).

response to some laboratory tests of Iksha pass L_6 used in the earlier trials with that of the pass $L_6Hu_1L_2$ virus used in the less successful later trial in children.

MATERIALS AND METHODS

Seed pools of the Iksha strain of Influenza A2 virus, at the passage levels L_6 and $L_6Hu_1L_2$ described above, were given a single further egg passage; 0·2 ml. of a 10^{-4} dilution of the seed pool was inoculated into the allantoic cavity of 11-day-old chick embryos; infected allantoic fluids were harvested after 42 hr. incubation at 35° C. and stored in 2 ml. vol. at −70° C. after clarification by low-speed centrifugation. All subsequent tests were on these first laboratory pools, unless otherwise indicated in the text.

Haemagglutination (HA) and haemagglutination-inhibition (HI) tests were performed by the WHO plastic plate method (WHO Expert Committee on Influenza, 1953); a 1% suspension of human group O cells was used except where otherwise stated. Rabbit antisera to various influenza viruses were as previously described (Hobson, 1966).

The kinetics of adsorption to and elution from red cells was studied by methods based on those of Lief & Henle (1958).

Infectivity titrations by conventional methods in the allantoic cavity of 11-day-old developing fertile eggs, or in isolated pieces of chorioallantoic membrane (Fazekas de St Groth & White, 1958) were carried out at various temperatures, using specially controlled incubators (Bedson & Dumbell, 1961).

Infectivity titrations in Swiss white mice were performed by methods previously described (Lindenmann, Lane & Hobson, 1962).

RESULTS

Growth in eggs at various temperatures

It is known that the highest temperature at which viruses will grow in tissue culture or eggs may vary characteristically from strain to strain and, with the poxviruses, these ceiling temperatures offer a useful means of characterization (Bedson & Dumbell, 1964). Differences in the ability of polioviruses to grow at high temperatures has been employed as a means of differentiating virulent from attenuated strains, and cryophilic variants of poliovirus have been shown to be of decreased virulence (Dubes & Wenner, 1957); similar findings with influenza viruses have been shown by Alexandrova & Smorodintsev (1965). Accordingly, the growth characteristics of the two pools of Iksha virus were examined at various temperatures of incubation.

After incubation of inoculated eggs at 35° C for 48 hr. both the pools L_6 and $L_6Hu_1L_2$ showed similar infectivity titres (approx. $10^{9.5}$ EID50/ml.); neither strain caused death or obvious lesions in the chick embryos within this time, and HA/EID50 ratios were similar for both pools.

A single dilution (10^{-4}) of each pool was inoculated into eggs, and after incubation at various temperatures from 25 to 42° C. for 16 hr., allantoic fluids were

harvested and titrated for infectivity in chorioallantoic membrane (CAM) cultures maintained at 35° C. for 48 hr. At temperatures between 35 and 39° C. both strains grew equally well, to final titres of $10^{8\cdot5}$–$10^{9\cdot0}$ EID50/ml. At lower temperatures, a progressive reduction of growth occurred; although titres were generally 0·3–1·0 $\log_{10}$ lower with $L_6Hu_1L_2$ than with L_6 virus over this range the behaviour of the two types of virus could not be clearly differentiated, and titres of $10^{1\cdot4}$ and $10^{1\cdot7}$ EID50/ml. respectively were found at the lowest temperature, 25° C. At higher temperatures of incubation, the growth of both strains was equally affected over the range 39–40° C., but the degree of inhibition was comparatively small (titres = $10^{6\cdot7}$ EID50/ml.). Between 40·5° and 42° C. it was difficult to obtain closely comparable results in different experiments, owing probably to the poor viability of chick embryos at these high temperatures; however, in all tests Iksha L_6 remained capable of growth to titres of approx. 10^4 EID50/ml. at 42° C., whereas $L_6Hu_1L_2$ virus grew erratically, and titres of individual eggs were usually lower than $10^{1\cdot5}$ EID50/ml. It seemed possible that growth of Iksha virus pools at 25–27° C. or at 40–42° C. might have led to the selection of variant fractions of the original uncloned virus populations. However, the virus progeny of the two strains grown at these extreme temperatures for up to 48 hr. showed no difference from the parent strains in their growth-temperature range, nor in any of the properties to be described below, and would thus appear to be randomly-selected survivors of the original virus pools.

Nutritional factors

Certain differences in nutritional requirements for the growth of different strains of influenza virus in chorioallantoic membrane cultures have been shown by Eaton and his colleagues (Daniels, Eaton & Perry, 1952: Eaton, Adler & Perry, 1953) and by Veeraraghavan, Kirtikar & Sreevalsan (1961). However, in the present experiments, the growth of Iksha viruses in CAM cultures was not affected by the substitution of medium 199 for the simple glucose-saline solution of Fazekas de St Groth & White (1958), the replacement of glucose by sodium pyruvate (Levine, Bond & Rouse, 1956) or the addition of folic acid, glycine and sodium molybdate (Veeraraghavan *et al.* 1961) and attempts to find nutritional differences between the two virus pools have not been pursued further at this stage.

Mouse infectivity

Neither of the virus pools caused any deaths or signs of infection after intracerebral inoculation. After intranasal instillation both L_6 and $L_6Hu_1L_2$ pools gave similar results. Deaths with gross pulmonary consolidation occurred within 3–6 days after inoculation of either undiluted or a 10^{-1} dilution of infected allantoic fluids; no deaths occurred in mice inoculated with dilutions of 10^{-2} or 10^{-3} but when the survivors were killed, 8 days later, scattered pulmonary lesions were found in most animals; higher dilutions gave no evidence of infection.

Emulsions of lungs obtained from infected mice were directly titrated for their virus content in further batches of mice and in eggs. The mouse ID50/EID50 ratio of the emulsions was closely similar to that of the original virus pools. Thus,

there was no evidence that variants of significantly enhanced virulence had been selected by single passage through mice.

Iksha viruses which had been passaged in eggs incubated at 27° C. showed mouse ID50/EID50 ratios similar to those of the parent strain.

Haemagglutination characteristics

The range of activity of influenza virus haemagglutinins against various species of red blood cell, their thermostability and sensitivity to mucoid inhibitors, and the reversibility of haemagglutination, probably as an effect of viral neuraminidase, may differ from strain to strain. These differences have been shown by Burnet (1951) to behave as heritable properties of certain virus strains in genetic recombination experiments. The L_6 and $L_6Hu_1L_2$ pools of Iksha virus were, therefore, examined to determine whether significant differences in the behaviour of their haemagglutinins could be found.

Both virus pools gave similar HA titres when tested with human red blood cells (RBC) of group O, A, B and AB or horse, sheep and fowl RBC at 4° C., room temperature or 37° C.; after standing for a further 2 hr. at these incubation temperatures, some disaggregation was found, but only in HA shields produced by the higher concentrations of virus, and only at 37° C. Mouse RBC were agglutinated equally well by either pool but the HA shields were unstable and almost complete reversion of agglutination occurred rapidly at room temperature and at 37° C.

A more quantitative study of the elution of virus from RBC revealed some difference between the two Iksha strains. Washed human group O RBC were added in a final concentration of 10% to 50 ml. volumes of fresh allantoic passages of L_6 and $L_6Hu_1L_2$ viruses, each diluted in saline to contain 320 HA units per 0·25 ml. After incubation for 1 hr. at 4° C., with constant stirring, the RBC were recovered by centrifugation in the cold at 1000 rev./min. for 10 min. and twice washed with cold saline. No detectable haemagglutinin remained in the original supernatant fluid or the saline washings. Each RBC mass was resuspended in 5 ml. M/50 phosphate buffered saline and incubated with stirring at 37° C.; samples taken at serial intervals were centrifuged briefly in warmed containers. The supernatant fluids of each sample were tested by HA titration for eluted virus. The results (Fig. 1) show that $L_6Hu_1L_2$ virus eluted faster and more completely than the L_6 passage.

The thermal resistance of the haemagglutinin of the two Iksha pools was compared by incubating undiluted infected allantoic fluids for various times at temperatures from 37° to 56° C., and subsequently titrating at room temperature for residual HA activity. The two virus pools differed in behaviour as shown in Fig. 2; HA of both pools was rapidly destroyed at 53–56° C. but at temperatures of 49–51° C. the HA of the L_6 pool was inactivated more quickly and completely than that of $L_6Hu_1L_2$ virus.

To exclude the possibility that these were merely chance variations between the two egg pools, it was decided to investigate several further pools of L_6 and $L_6Hu_1L_2$ virus obtained by passage at or near their terminal infective dilutions. Groups of 11-day-old chick embryos were inoculated allantoically with 0·2 ml. of a 10^{-8} or

10^{-9} saline dilution of L_6 or $L_6Hu_1L_2$ pool. Allantoic fluids were harvested separately after 42 hr. incubation at 35° C. and tested for haemagglutinin. Samples of each positive fluid were retitrated after being incubated at 49·5° C. for 30 min. and 50° C. for 60 min. The results (Table 1) confirmed the sharp differences in thermal stability of HA of the two virus strains. In many cases, heating at 49·5° C. for

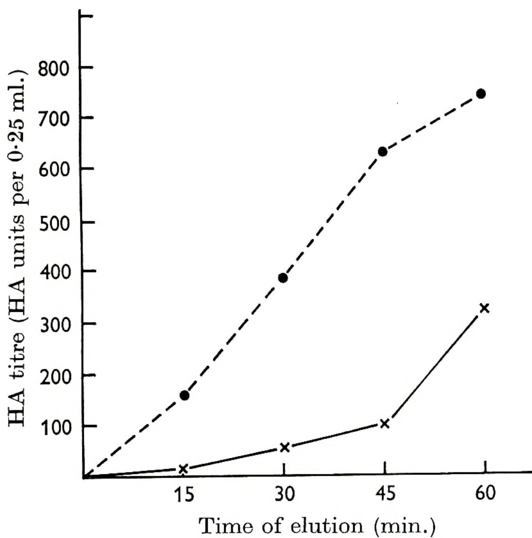


Fig. 1

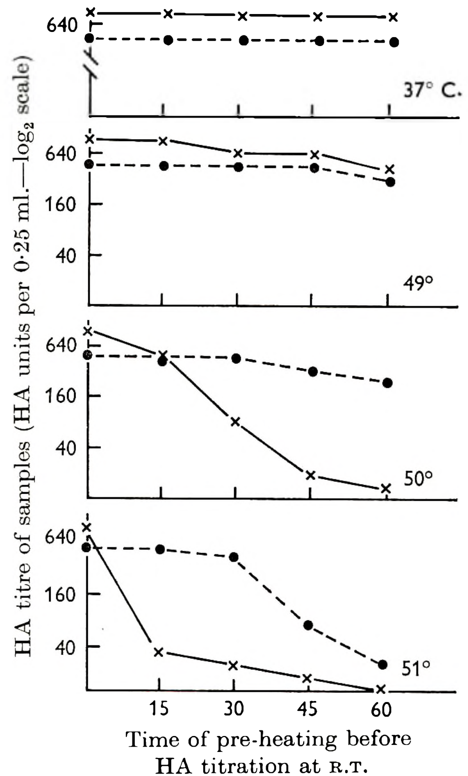


Fig. 2

Fig. 1. The rate of elution of two pools of Iksha influenza virus from haemagglutinated human RBC ×—×, Early pass, L_6; ●---●, later pass, $L_6Hu_1L_2$.

Fig. 2. The effect of heat on the HA titre of two pools of Iksha influenza virus. ×—×, Early pass, L_6; ●---●, later pass, $L_6Hu_1L_2$.

30 min. gave a rise in HA titre, possibly as a result of virus disaggregation or removal of non-specific inhibitors in the fluid; on continued incubation of the HA tests at room temperature, shields of agglutination produced by heated L_6 virus rapidly crumbled whereas HA shield-patterns produced by heated $L_6Hu_1L_2$ were stable; no dissociation occurred in titrations of fresh virus pools of either strain, and it was presumed that heating had affected the strength of bonding of virus to RBC. At 50° C. for 60 min. 0/7 pools of L_6 showed more than a trace of residual HA, whereas 6/7 pools of $L_6Hu_1L_2$ were virtually unaffected by this temperature.

The stock pools of L_6 and $L_6Hu_1L_2$ virus were tested for HI at a constant dosage of 8 HA units against various animal sera. There was no inhibition of haemag-

glutination by normal horse, mouse or rabbit serum or by specific rabbit antisera against A (Swine), AO (WS and PR8) or A1 (FM1) strains of influenza virus. With standard antiserum against A2/Jap/305/57/EFME, HI titres were 1/4096 against the homologous virus, 1/1024 against L_6 virus and 1/512 against the $L_6Hu_1L_2$ strain. A rabbit antiserum prepared against L_6 virus showed an HI titre of 1/320 against the same strain, but 1/192 against $L_6Hu_1L_2$ virus. It would thus appear that the avidity of the two virus strains for antibody differs, or that there may be slight differences in antigenic constitution. HI tests with each of the terminal dilution pools of each virus showed that all those derived from L_6 were more sensitive to inhibition by the antiserum prepared against L_6 virus than were those derived from the $L_6Hu_1L_2$ strain.

Table 1. *The thermostability of HA of various limiting-dilution clones of two pools of Iksha influenza* A_2 *virus*

Parental virus	Clone no.	HA titres of allantoic fluid suspensions of virus clones: Unheated	After heating: 49·5°/30 min.	After heating: 50°/60 min.	Stability of HA of heated virus
Early pass, L_6	− 8, a	480	320	20	—
	− 8, b	1920	1280	20	—
	− 8, c	480	320	20	—
	− 8, d	960	1280	20	—
	− 9, e	960	960	< 20	—
	− 9, f	100	< 20	< 20	
	− 9, g	1920	< 20	< 20	
Later pass, $L_6Hu_1L_2$	− 8, a	240	240	480	+
	− 8, b	960	1280	640	+
	− 8, c	240	240	160	+
	− 8, d	480	960	1280	+
	− 8, e	320	480	240	+
	− 9, f	320	640	640	+
	− 9, g	960	1280	60	+

— = HA titration showing disaggregation of RBC shields after a further 1–2 hr. at R.T.

DISCUSSION

The aim of the present experiments was to assess certain laboratory characteristics of two different pools of the Russian A2 virus Iksha, one of which ($L_6Hu_1L_2$) appeared to be more attenuated than the other (L_6), in the sense that fewer infections and a lesser serological response could be demonstrated after inoculation into volunteers. As would be expected, the two close passage levels of this single virus strain did not differ in most respects. However, the more 'virulent' pool, i.e. the L_6 strain used fairly successfully in clinical trials, could be differentiated to some extent by laboratory tests. The HA of L_6 virus was less thermostable than that of the later passage $L_6Hu_1L_2$, and terminal dilution clones derived from each strain behaved similarly to the uncloned parent pools. The rate of elution of virus from human RBC was slower for L_6 than for $L_6Hu_1L_2$, and this may

indicate differences in the neuraminidase activity of the two strains; however, both were fully resistant to non-specific serum inhibitors of haemagglutination. In HI tests with specific Asian antisera L_6 was slightly more sensitive than the later passage, but serological cross-adsorption studies would be required before this could be assumed to be a true antigenic difference. The L_6 pool showed a slightly greater capacity to multiply in the chorioallantoic membrane at temperatures above 40° C. than the apparently more attenuated $L_6Hu_1L_2$ pool. Recently, Alexandrova & Smorodintsev (1965) have shown that cryophilic variants of certain A2 and B strains of influenza virus, which are incapable of growth at 38°–40° C., are of lower human virulence than the original parent strains.

The precise definition of genetic markers which may be associated with the infectivity of influenza viruses for man has, however, been limited by the lack of suitable experimental animal infections which are a realistic model of the human disease, and by the lack of tissue culture plaque techniques with a sufficiently high plating efficiency for exact quantitative studies. However, Burnet (1951) showed that, with respect to neurovirulence for mice, the avirulent AO strain WSM could be differentiated from the virulent variant NWS by several laboratory markers, particularly by the reduced thermostability of HA of the neurotropic strain. More recently, Soloviev, Orlova, Porubel & Vasilieva (1961) compared the behaviour of egg-grown strains of A2 influenza virus of known difference in human virulence. In general, moderately attenuated strains which were acceptable as living vaccines showed greater HA-thermostability than strains of greater virulence, which had produced untoward reactions on inoculation into volunteers. The attenuated strains also eluted more readily from red blood cells, were less sensitive to non-specific HA-inhibitors and gave higher HA titres with mouse and horse RBC than the virulent strains. It cannot be determined whether these were merely chance differences between unrelated virus strains or whether they were correlated in any way with the observed differences in virulence.

In the present experiments, the two virus pools L_6 and $L_6Hu_1L_2$ were derived from a single parent strain with only a few intervening steps. Here it is perhaps more reasonable to assume that their differences in laboratory behaviour may in fact be due to modifications of genetic constitution, and that these changes may be linked in some way with the observed change in clinical performance. The range of virulence of L_6 and $L_6Hu_1L_2$ (i.e. from satisfactory attenuation to over-attenuation) is below the range investigated by Soloviev *et al.* (1961), but the trend of increasing thermostability and increasing ease of elution with decreasing infectivity for man is similar to that shown by the Russian workers.

However, influenza viruses are notoriously labile on laboratory manipulation, and it has yet to be shown that the different properties of L_6 and $L_6Hu_1L_2$ will breed true over controlled serial passage. Before any significant relationship could be assumed between these markers and virulence, it would be necessary to show that clones of virus with the laboratory markers of $L_6Hu_1L_2$ could be segregated from the L_6 pool and subsequently shown to be avirulent, or conversely that L_6-like virus could be segregated from the $L_6Hu_1L_2$ pool and shown to have regained virulence. It would also be of interest to study the changes in genetic markers of

further influenza viruses over the whole course of their development from 'wild-type' strains to living vaccine strains of clinically satisfactory attenuation.

SUMMARY

Two pools of living influenza vaccine derived from the Russian A2 strain Iksha had given discrepant results in clinical trials. The less effective pool showed an increased thermostability of haemagglutinin, an increased elution rate from red blood cells and a reduced ability to grow in chorioallantoic membranes at temperatures above 40° C. The relationship of these findings to the virulence of influenza viruses is discussed.

REFERENCES

Alexandrova, G. I. & Smorodintsev, A. A. (1965). Obtaining of an additionally attenuated vaccinating cryophil influenza strain. *Rev. roum. Inframicrobiol.* **2**, 179.

Andrews, B. E., Beare, A. S., McDonald, J. C., Zuckermann, A. J. & Tyrrell, D. A. J. (1966). Further trials of live influenza vaccine. *Br. med. J.* i, 637.

Beare, A. S., Tyrrell, D. A. J., McDonald, J. C., Pollock, T. M., Taylor, C. E. D., Howells, C. H. L. & Tyler, L. E. (1967). Reactions and antibody response to live influenza vaccine prepared from the Iksha (A2) strain. *J. Hyg., Camb.* **65**, 245.

Bedson, H. S. & Dumbell, K. R. (1961). The effect of temperature on the growth of pox viruses in chick embryo. *J. Hyg., Camb.* **59**, 457.

Bedson, H. S. & Dumbell, K. R. (1964). Hybrids derived from viruses of alastrim and rabbit pox. *J. Hyg., Camb.* **62**, 141.

Burnet, F. M. (1951). A genetic approach to variation in influenza viruses. 1. The characters of three substrains of influenza virus A (WS). *J. gen. Microbiol.* **5**, 46.

Daniels, J. B., Eaton, M. D. & Perry, M. E. (1952). Effect of glucose on the growth of influenza virus in deembryonated eggs and tissue cultures. *J. Immun.* **69**, 321.

Dubes, G. R. & Wenner, H. A. (1957). Virulence of polioviruses in relation to variant characteristics distinguishable on cells *in vitro*. *Virology* **4**, 275.

Eaton, M. D., Adler, L. T. & Perry, M. E. (1953). Virus growth and cellular energy production: effect of substances chemically related to thyroxin on influenza virus. *Proc. Soc. exp. Biol. Med.* **84**, 57.

Fazekas de St Groth, S. & White, D. O. (1958). An improved assay for the infectivity of influenza viruses. *J. Hyg., Camb.* **56**, 151.

Hobson, D. (1966). The strain-specific serological activity of a non-haemagglutinating fraction of influenza viruses. *Br. J. exp. Path.* **47**, 257.

Levine, A. S., Bond, P. H. & Rouse, H. C. (1956). Modification of viral synthesis in tissue culture by substituting pyruvate for glucose in the medium. *Proc. Soc. exp. Biol. Med.* **93**, 233.

Lief, F. S. & Henle, W. (1956). Studies on the soluble antigen of influenza virus. II. A comparison of the effects of sonic vibration and ether treatment of elementary bodies. *Virology* **2**, 772.

Lindenmann, J., Lane, C. A. & Hobson, D. (1963). The resistance of A2G mice to myxoviruses. *J. Immun.* **90**, 942.

McDonald, J. C., Zuckermann, A. J., Beare, A. S. & Tyrrell, D. A. J. (1962). Trials of live influenza vaccine in the Royal Air Force. A Report to the Medical Research Council Committee on Influenza and Other Respiratory Virus Vaccines. *Br. med. J.* i, 1036.

Soloviev, V. D., Orlova, T. G., Porubel, L. A. & Vasilieva, I. N. (1961). Study of the genetic characters of vaccine strains of type A-2 influenza virus. *Vop. virus* **6**, 684.

Veeraraghavan, N., Kirtikar, M. W. & Sreevalsan, T. (1961). Studies on the cultivation of influenza virus *in vitro*. *Bull. Wld Hlth Org.* **24**, 711.

World Health Organization expert committee on influenza. (1953). *Tech. Rep. Ser. Wld Hlth Org.* no. 64.

The Quarterly Journal of Medicine

Edited by D. H. BRINTON, W. D. W. BROOKS, R. B. HUNTER,
J. D. N. NABARRO, M. L. ROSENHEIM, A. G. W. WHITFIELD

CONTENTS

NEW SERIES, Vol. XXXV No. 142. APRIL 1967

(Vol. LIX of the continuous series)

Sterile Blood Cultures in Bacterial Endocarditis. By J. R. Hampton and M. J. G. Harrison.

Genetic and Non-Genetic Factors in Simple Goitre Formation: Evidence from a Twin Study. By William R. Greig, James A. Boyle, Anne Duncan, Janette Nicol, Mary J. B. Gray, W. Watson Buchanan and Edward M. McGirr.

Dementia and Folate Deficiency. By R. W. Strachan and J. G. Henderson.

Ischaemic Lesions of the Spinal Cord: An Illustrated Review. By R. A. Henson and M. Parson.

Hypertrophic Neuropathy. By P. K. Thomas and R. G. Lascelles. With plates 13 to 17.

Pulmonary Infections and Rheumatoid Arthritis. By W. C. Walker.

A Syndrome of Loin Pain and Haematuria Associated with Disease of Peripheral Renal Arteries. By P. J. Little, J. S. Sloper and H. E. de Wardener.

Single Numbers 20*s.* net each   Subscription 60*s.* per annum

OXFORD UNIVERSITY PRESS

Ely House, 37 Dover Street, W. 1

The Journal of General Microbiology

Editors: B. C. J. G. Knight and A. F. B. Standfast

Contents of Vol. 47, No. 1   April 1967

A. H. Reisner and H. Macindoe. Incorporation of amino acid into protein by utilizing a cell-free system from Paramecium.

M. Hutchinson, K. I. Johnstone and D. White. Taxonomy of anaerobic thiobacilli.

M. Felton, Jun., J. J. Cooney and W. G. Moore. A quantitative study of the bacteria of a temporary pond.

B. Jarvis. Resistance to nisin and production of nisin-inactivating enzymes by several Bacillus species.

J. D. Anderson and G. T. Crouch. A new principle for the determination of total bacterial numbers in populations recovered from aerosols.

P. N. Hobson and R. Summers. The continuous culture of anaerobic bacteria.

S. G. Wilkinson. The sensitivity of pseudomonads to ethylene diaminetetra-acetic acid.

P. Weglenski. The mechanism of action of proline suppressors in *Aspergillus nidulans.*

W. J. Brammar, P. H. Clarke and A. J. Skinner. Biochemical and genetic studies with regulator mutants of the *Pseudomonas aeruginosa* 8602 amidase system.

A. Afshar. The growth of *Mycoplasma bovigenitalium* in cell cultures.

A. B. Harris. Inhibition of growth and nucleic acid synthesis in iron-deficient *Mycobacterium smegmatis.*

W. A. Hamilton and J. M. Stubbs. Comparison of the germination and outgrowth of spores of *Bacillus cereus* and *Bacillus polymyxa.*

J. T. Smith. Production of thymineless mutants in gram-negative bacteria (Aerobacter Proteus).

H. U. Joshi, A. J. H. Carr and D. G. Jones. Effect of clover phyllody virus on nodulation of white clover (*Trifolium repens*) by *Rhizobium trifolii.*

H. Williams Smith and S. Halls. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production.

The subscription rate is £5 net per volume of 3 parts. Single parts 45s. each, plus postage. Four volumes will be issued each year. Orders should be sent to

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1

Journal of Hygiene Volume 65, No 2. June 1967

CONTENTS

(*All rights reserved*)

PAGE

MEYNELL, G. G. and WILLIAMS, TREVOR. Estimating the date of infection from individual response times 131

BHASKARAN, K. and SINHA, V. B. Attenuation of virulence in *Vibrio cholerae* 135

HUANG, C. T. and LO, C. B. Human infection with *Salmonella choleraesuis* in Hong Kong 149

FARRELL, I. D. and ROBERTSON, L. The sensitivity of the biotypes of *Brucella abortus* to three antibiotics used in selective media, and the description of a new biotype 165

WILDY, PETER. The progression of herpes simplex virus to the central nervous system of the mouse 173

LIDWELL, O. M., RICHARDS, I. D. G. and POLAKOFF, S. Comparison of three ventilating systems in an operating room 193

WILLIAMS, R. E. O. Airborne staphylococci in the surgical ward . . 207

JELLARD, C. H. and CHURCHER, GILLIAN M. An outbreak of *Pseudomonas aeruginosa* (*pyocyanea*) infection in a Premature Baby Unit, with observations on the intestinal carriage of *Pseudomonas aeruginosa* in the newborn 219

PHILLIPS, IAN. *Pseudomonas aeruginosa* respiratory tract infections in patients receiving mechanical ventilation 229

HARVEY, R. W. S. and PRICE, T. H. The isolation of salmonellas from animal feedingstuffs 237

BEARE, A. S., TYRRELL, D. A. J., MCDONALD, J. C., POLLOCK, T. M., TAYLOR, C. E. D., HOWELLS, C. H. L. and TYLER, L. E. Reactions and antibody response to live influenza vaccine prepared from the Iksha (A2) strain . 245

HOBSON, D., GOULD, E. A. and FLOCKTON, HILARY I. Laboratory studies on a strain of Asian influenza virus used as a living vaccine . . . 255

The Journal of Hygiene is issued in four parts to a volume.

Quotations can be given for back volumes on application.

Papers for publication should be sent to Dr R. M. Fry, Department of Pathology, Tennis Court Road, Cambridge. Other communications should be addressed to the University Printing House, Cambridge.

Papers forwarded to the Editor for publication are understood to be offered to *The Journal of Hygiene* alone, unless the contrary is stated.

Contributors receive twenty-five copies of their paper free. Additional copies may be purchased; these should be ordered when the final proof is returned.

The subscription price is £4. 10*s*. per volume (post free), payable in advance; single numbers 32*s*. 6*d*. net. Subscriptions may be sent to any Bookseller, or to The Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1.

The subscription price in the U.S.A. is $17.50. Single parts $5.00. Enquiries should be addressed to the American Branch of the Press at 32 East 57th Street, New York, N.Y. 10022.