Vol. 67, No. 1 March 1969

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

P. ARMITAGE, M.A., PH.D.

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., F.R.C.P.

E. T. C. SPOONER, C.M.G., 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. net* ($15.50) (4 parts)

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

The Journal of Hygiene

Papers for publication should be sent to Dr R. M. Fry, Department of Pathology, Tennis Court Road, 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.

SUBSCRIPTIONS

The Journal of Hygiene is published quarterly.

Single parts 32*s*. 6*d*. net ($5.00 in the U.S.A.) plus postage.

Four parts form a volume. The subscription price of a volume (which includes postage) is £4 10*s*. net ($15.50 in the U.S.A.).

Second class postage paid at New York, N.Y.

Quotations for back volumes may be obtained from the publisher.

Orders should be sent to a bookseller or to the publisher:

Cambridge University Press
Bentley House, 200 Euston Road, London, N.W. 1
32 East 57th Street, New York, N.Y. 10022

Corrigendum

F. W. WINTON and A. J. KEAY. *J. Hyg., Camb.* (1968) **66**, 325–42

On page 330, line 7;

For 28 out of 210 nurses' hands (13·4 %)

Read 28 out of 70 nurses' hands (40·0%).

ห้องสมุด กรมวิทยาศาสตร์
2512

J. Hyg., Camb. (1969), **67**, 1
Printed in Great Britain

Live influenza B vaccine in volunteers

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

D. HOBSON
Bacteriology Department, University of Liverpool

C. H. L. HOWELLS
Virus Laboratory, New Cross Hospital, Wolverhampton

M. S. PEREIRA AND T. M. POLLOCK
Central Public Health Laboratory, Colindale, London, N.W.9

AND L. E. TYLER
General Practitioner, Wolverhampton

(*Received* 20 *December* 1967)

In this paper we describe our experience with a live vaccine made from an influenza type B virus. Type B was chosen for a number of reasons. We had had no previous experience with it, an attenuated virus with an acceptable passage history was available, and the low incidence of haemagglutination-inhibiting (HI) antibodies in the population was likely to make it easier to find susceptible volunteers than when an A virus was used. In addition, because different antigenic subtypes of influenza B circulate at the same time (Communicable Disease Center Report, 1965) we wished to look at the evidence for cross-protection. We also wished to assess the importance of repeated vaccinations, on which some Russian workers lay considerable stress (Smorodintsev *et al.*, 1965).

Previous trials of live influenza vaccines in Great Britain (in which repeated vaccinations had not been given) had used vaccines made from a 1957 Russian A 2 virus (McDonald *et al.* 1962; Andrews *et al.* 1966; Beare *et al.* 1967). The results showed that attenuated live viruses infected fairly readily when serum antibody was low or absent, that infection was followed by resistance to challenge with the same virus, and that there was a significant rise of antibody in a proportion of people.

* Members of Committee: Prof. C. H. Stuart-Harris (*Chairman*), Dr B. E. Andrews, Dr A. S. Beare, Prof. G. Belyavin, Dr J. T. Boyd, Prof. G. W. A. Dick, Prof. Sir Austin Bradford 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 and Dr T. M. Pollock (*Secretary*).

MATERIALS AND METHODS

Virus

The influenza B virus was derived from throat swabs from which the virus had previously been isolated and which had been stored at $-40°$ C. Fluid from bottles containing the swabs was inoculated into the allantoic and the amniotic cavities of fertile hens' eggs, which were guaranteed free of avian leucosis and of mycoplasma and which had been incubated for 10 or 11 days. An agent known as B/England/13/65 was later chosen for serial passage. Haemagglutinin was detected both in the allantoic and in the amniotic fluids on first isolation, and passages at 10^{-4} and 10^{-5} dilutions were made from the allantoic fluid in groups of leucosis free eggs. Pools of virus were dispensed in 1·0 ml. amounts and stored in sealed glass ampoules at $-65°$ C.

The virus used in the trial was taken from a pool which had had six egg passages and which contained $10^{8\cdot7}$ fifty-per-cent-egg-infecting doses (EID50) in 0·1 ml. It agglutinated 0·5 % fowl red cells to a titre of 1/320.

Testing of the vaccine for safety

Safety tests were performed to exclude the possibility of accidental contamination with bacteria and other viruses (McDonald *et al.* 1962).

Volunteers for trial.

One hundred and sixty-four employees of Sankey Ltd., Wolverhampton, were enrolled for vaccination. They were questioned by one of us about recent and chronic respiratory disease and about their general health. Apart from the elimination of a few after questioning they were unselected, were of both sexes and ranged in age from 17 to 65 years. It had been hoped to arrange for an unvaccinated control group, which would be observed for cases of any infectious respiratory disease that might break out in the vicinity, but this proved impracticable because additional volunteers were not available.

In the trial later held at the Common Cold Research Unit nine volunteers were inoculated, of whom five were male and four female. They were all healthy adults under 40.

Administration of the vaccine

Before each vaccination session the virus was diluted to $10^{-4\cdot0}$ in Hanks's balanced salt solution (BSS) with 0·2 % bovine plasma albumin (BPA) and adjusted to a pH of about 7·2. A coarse hand-spray (Fig. 1) was used to administer the vaccine, which was inoculated into both nostrils. The dose of virus given to each individual was 0·5–0·6 ml.—that is, about $10^{5\cdot4}$ EID50. Ampoules from a single pool of virus were used throughout the trial.

Three vaccinations were given, at intervals of 3 weeks, in the autumn of 1966. Most volunteers attended for all the vaccinations, but some for only one or two. In June 1967, 7 months after the third vaccinations, as many of the original

volunteers as were still available were challenged with the vaccine virus in an attempt to assess their long-term resistance to infection.

At the Common Cold Research Unit a single dose of the virus was given intranasally from a measured dropper. The same pool of virus was used as in Wolverhampton.

Collection of blood for antibody estimations

Volunteers were bled six times—immediately before each of the first three vaccinations, 3 weeks after the third vaccination, immediately before the challenge dose and about 3 weeks after that. At Salisbury, volunteers were bled before the trial and 2–3 weeks afterwards.

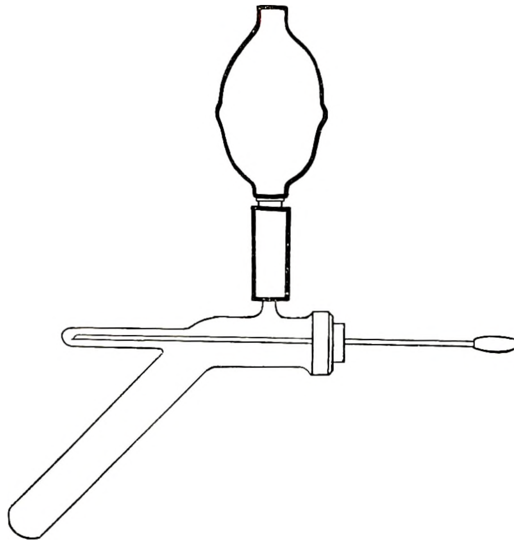


Fig. 1. Spray used in the vaccination of volunteers. It was copied from a model provided by Prof. A. A. Smorodintsev of the U.S.S.R. Academy of Medical Sciences, Leningrad.

Virus isolation after vaccination

In Wolverhampton, throat swabs were taken 72 hr. after vaccination, and in the Common Cold Research Unit nasal washings were taken 2, 3 and 4 days after virus inoculation. The specimens were at both places inoculated into primary cultures of monkey kidney which were tested for haemadsorption after 5 days incubation in a rolling drum at 33° C.

Measurement of antibodies

Serum antibodies were measured by haemagglutination-inhibition (World Health Organization, 1953) and by the strain-specific complement fixation test (VCF) (Lief & Henle, 1956; Pereira *et al.* 1967).

Neutralization tests at Salisbury were performed as described previously (Beare, 1962). The microtitre method for the estimation of HI antibody titres against other subtypes of influenza B was that of Sever (1962). Tests for antibody on the surface of the nasal mucosa were as described by Smith *et al.* (1966).

Recording of clinical symptoms

Volunteers at Wolverhampton reported their symptoms to the sick bay at the factory and were questioned when they attended for the next vaccination. Symptoms were easily recognizable and histories, although subjective, were probably mainly reliable. A degree of error was, however, probably inherent in the system and it seems likely that the incidence of reactions tended to be overestimated. At the time of the fourth vaccination each volunteer was given a simple questionnaire in place of the interview that followed the earlier vaccination sessions.

RESULTS

The effects of vaccination were judged by virus isolation, rises of circulating antibody and clinical reactions. Table 1 provides a general summary of results. Virus recoveries were almost certainly never complete but the same technique

Table 1. *Frequency of virus isolations, antibody rises and clinical reactions after repeated vaccination with B/England/13/65*

(Numerators denote responses, denominators numbers tested.)

Vaccination	Virus isolations		Antibody rises: HI		Antibody rises: VCF		Incidence of clinical reactions	
First	29/135	21 %	43/128	34 %	18/66	27 %	85/121	70 %
Second	8/113	7 %	10/103	10 %	3/65	5 %	38/101	38 %
Third	0/70	0 %	1/76	1 %	2/65	3 %	5/61	8 %
Challenge	0/29	0 %	0/39	0 %	Not done		10/39	26 %

HI = haemagglutination-inhibition, VCF = strain specific complement-fixation.

Table 2. *Virus isolation in relation to serum antibody before each vaccination*

(Numerators denote responses, denominators numbers tested.)

Antibody (and range of titre)	Virus isolations: After the 1st vaccination		After the 2nd vaccination		After the 3rd vaccination		After the challenge	
HI < 6–24	25/113	22 %	4/59	7 %	0/34	0 %	0/20	0 %
> 24	4/22	18 %	4/54	7 %	0/36	0 %	0/9	0 %
VCF < 8–16	9/60	15 %	3/39	8 %	0/36	0 %	Not done	
> 16	2/5	40 %	1/27	4 %	0/24	0 %	—	

HI = haemagglutination-inhibition, VCF = strain-specific complement-fixation. Sera for VCF were available from only a proportion of volunteers.

was adopted after each vaccination and it is probably fair to compare them. The reduction in virus isolations with successive vaccinations was notable. They dropped from 21 % after the first administration to nil after the third. When the challenge was made 7 months later there were again no isolations; five volunteers

Table 3. *Antibody formation after vaccination, measured by haemagglutination-inhibition and by strain specific complement-fixation*

(Numerators are numbers who responded, denominators numbers of specimens tested.)

Range of antibody titres before each vaccination	Frequency of antibody rises after each vaccination							
	First		Second		Third		Challenge	
HI								
< 6–24	42/102	41 %	7/59	12 %	1/37	3 %	0/26	0 %
> 24	1/26	4 %	3/44	6 %	0/39	0 %	0/13	0 %
VCF								
< 8–16	18/61	30 %	3/40	7 %	2/37	5 %	Not tested	
> 16	0/5	0 %	0/25	0 %	0/28	0 %		

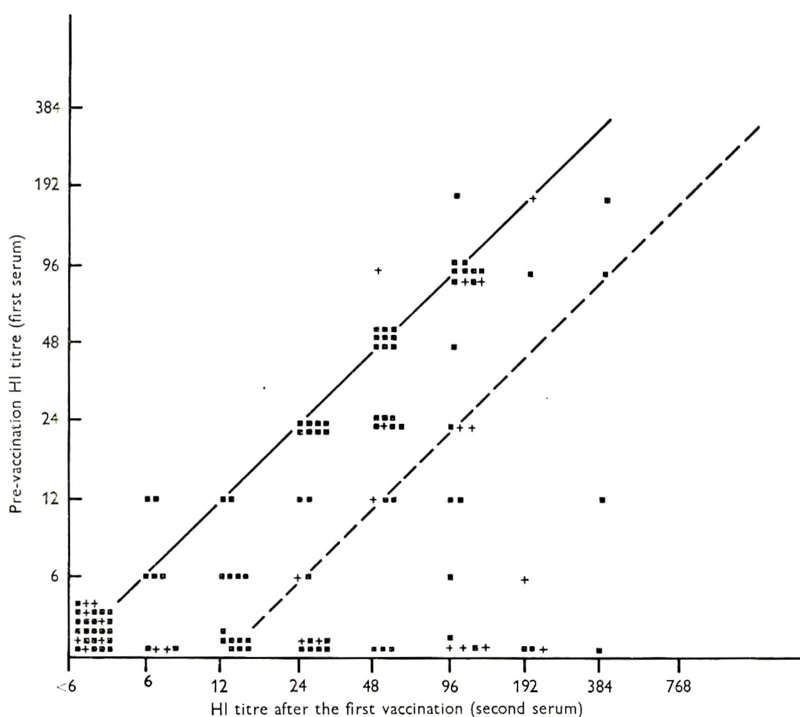


Fig. 2. The effect on HI antibody of the first dose of the vaccine. The ordinate represents HI titres before the trial and the abscissa the HI titres 3 weeks after vaccination. +, A volunteer from whom virus was recovered. The continuous line marks the line of no change in titre, the dotted line that of a fourfold rise in titre.

who were vaccinated for the first time on that occasion yielded one isolation. Antibody rises occurred mostly in those who had had little or no antibody at the beginning of the trial and the two tests measured them with differing sensitivity. The commonest clinical reaction was afebrile coryza lasting about 3 days; a very small percentage of volunteers had a mild influenza-like illness and some had no symptoms at all. Reactions were recorded for 70 % of the volunteers receiving the first virus dose; they fell to 8 % after the third and rose again to 26 % after the

challenge. It seems likely that the incidence of reactions reflected the development of immunity and its waning with the passage of time, but it is also clear that our estimates of reactions nearly always included some false positives.

The relationship between initial titres of HI and VCF antibody and virus isolation is shown in Table 2. Those with low titres were not conspicuously more susceptible to infection than those with high titres; and after the second and third vaccinations both groups were resistant.

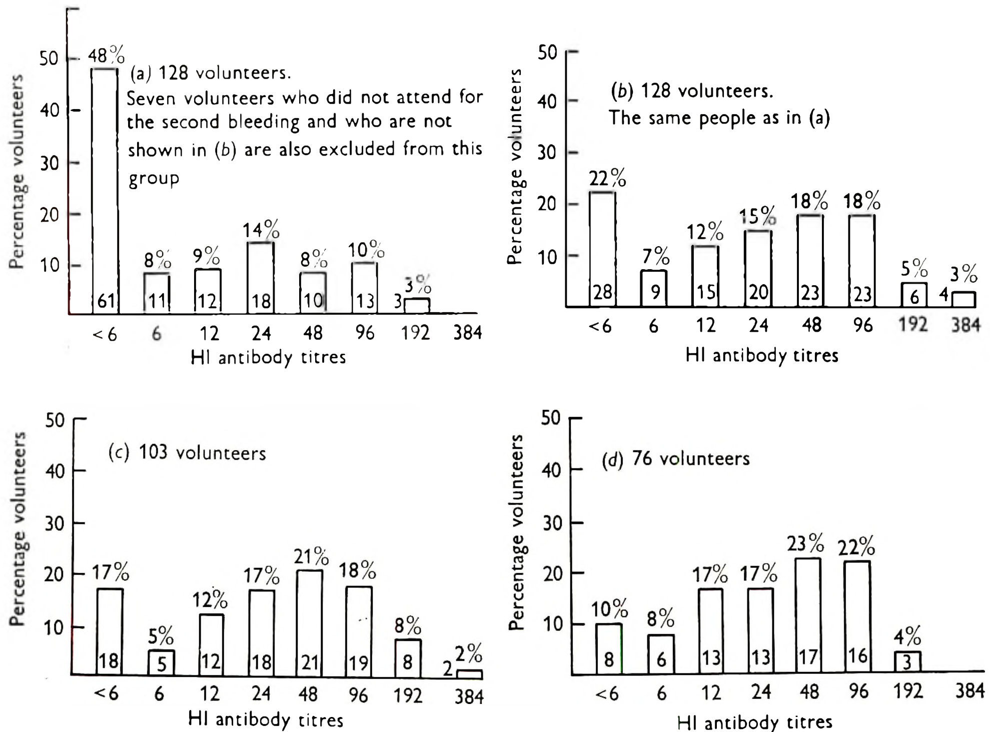


Fig. 3. Volunteers according to serum HI antibodies at different stages of the trial. (*a*) Immediately before the first vaccination (first serum). (*b*) Three weeks after the first vaccination (second serum) and immediately before the second vaccination. (*c*) Three weeks after the second vaccination (third serum) and immediately before the third vaccination. (*d*) Three weeks after the third vaccination (fourth serum).

Rises of antibody after the vaccinations are illustrated in Figs. 2–4 and in Table 3. The highest rises of titre came after the first vaccination and were most notable in people with low initial titres. But the scale of the rises (Fig. 2) rarely matched those stimulated by a killed vaccine. A minority of people did not respond at all and some of these had been shown to be excreting virus.

The modifying effect of serum antibody on clinical reactions is shown in Table 4. Seventy-four per cent of people with low HI antibody titres experienced a reaction after the first vaccination, but only 15 % after the third. This trend is reproduced in the VCF results and suggests a protective influence independent of measurable circulating antibody.

HI antibody estimations were performed on sixty of the original volunteers

7 months after the third vaccination. Fifty-two (87 %) had maintained their antibody titres while eight (13 %) showed a significant fall. It is noteworthy that there were no antibody rises after the challenge.

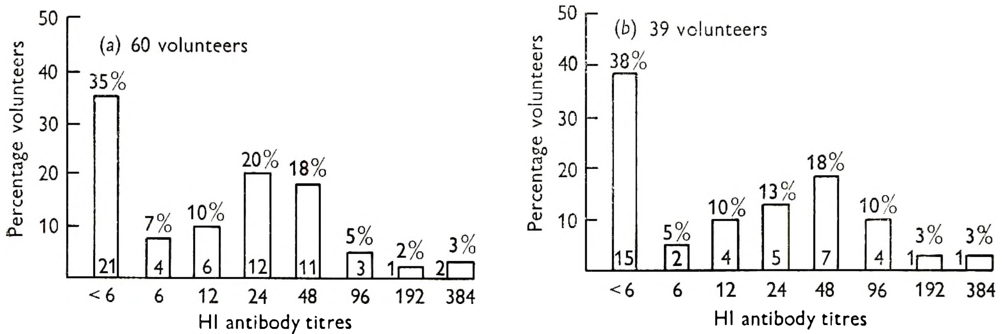


Fig. 4. The serological state of the volunteers in the follow-up period. (*a*) Seven months after the third vaccination and immediately before the challenge. (*b*) Twenty days after the challenge.

Table 4. *Clinical reactions in relation to serum antibody titres before each vaccination*

(The reactions were classified as follows: 0, no symptoms; I, coryza; II, coryza with malaise; III, mild influenza-like reactions.)

Range of titre before each vaccination	Incidence of different grades of clinical reaction after the vaccinations (%)				Numbers providing data
	0	I	II	III	
(*a*) HI antibodies					
< 6–24					
Before:					
1st vaccination	26	35	36	3	98
2nd vaccination	63	26	11	0	57
3rd vaccination	85	12	3	0	34
Challenge	75	21	4	0	24
> 24					
Before:					
1st vaccination	65	13	18	4	23
2nd vaccination	61	23	16	0	44
3rd vaccination	96	4	0	0	27
Challenge	73	20	7	0	15
(*b*) VCF antibodies					
< 8–16					
Before:					
1st vaccination	27	33	35	5	57
2nd vaccination	75	19	6	0	31
3rd vaccination	92	4	4	0	26
> 16					
Before:					
1st vaccination	40	20	40	0	5
2nd vaccination	46	31	23	0	26
3rd vaccination	100	0	0	0	18

VCF tests were not performed on sera collected before or after the challenge.

It was thought desirable to investigate the likelihood of heterotypic responses after infection and especially after multiple vaccinations. The antigenic position of the virus was established by Dr H. G. Pereira of the World Influenza Centre

Table 5. *Relationship of clinical reactions (all grades) and laboratory evidence of infection (virus isolation or antibody increase or both)*

(Numerators show clinical reactions, denominators numbers who provided data.)

Vaccination	Frequency of reactions							
	Virus isolated		Virus not isolated		Antibody rise		No antibody rise	
First	21/25	84 %	64/96	67 %	38/43	88 %	42/71	59 %
Second	3/5	60 %	34/94	36 %	6/13	46 %	23/76	30 %
Third	0/0	—	5/60	8 %	0/4	0 %	5/53	9 %
Challenge	0/0	—	9/36	25 %	0/0	—	6/25	24 %

Table 6. *Antigenic relationship of the vaccine to other influenza type B viruses*

(Table provided by Dr H. G. Pereira of the World Influenza Centre.)

Strain	Haemagglutination-inhibition test (Ferret Sera)				
	B/Johannesburg/33/58	B/Amakusa/1/64	B/Singapore/3/64	B/Eng/13/65	B/Rome/1/67
B/Johannesburg/33/58	**960**	60	120	40	< 10
B/Amakusa/1/64	80	**480**	160	120	60
B/Singapore/3/64	80	20	**960**	480	150
B/England/13/65	60	60	480	**960**	480
B/Rome/1/67	15	20	160	240	**480**

Table 7. *Antibody responses to related and rare influenza B viruses after repeated vaccination with B/England/13/65 (see Table 6)*

(The incidence of heterotypic responses in twelve volunteers who had developed antibody rises to the vaccine virus (*a*) is compared with a similar number who had not responded (*b*). Antibodies were measured by HI by the microtitre method of Sever (1962). There was a fourfold rise of HI antibody against the viruses shown.)

	B/England/13/65		B/Johannesburg/33/58		B/Amakusa/1/64		B/Singapore/3/64	
(*a*)	12	100 %	8	67 %	9	75 %	6	50 %
(*b*)	0	0 %	2	17 %	0	0 %	0	0 %

	B/Rome/1/67		B/Taiwan/2/62		B/India/363/64	
(*a*)	3	25 %	6	50 %	4	33 %
(*b*)	0	0 %	1	8 %	0	0 %

(Table 6) and the incidence of fourfold antibody rises in a few available sera is illustrated in Table 7. All the volunteers who had responded to the vaccine showed rises in antibody against some other influenza type B viruses, even against those

distantly related and even against B/Taiwan/2/62 and B/India/363/64 which have made only rare and circumscribed appearances as agents of natural infection. This small series did not reveal a broadening of the antibody spectrum after the later vaccinations such as was shown by Henle & Lief (1963) with the influenza A viruses in animals.

Finally, the many pointers to protective influences other than those mediated by circulating antibodies prompted a limited investigation on nine volunteers at the Common Cold Research Unit, into the possible role of local antibody. Five of these had no circulating antibody when tested by HI and by neutralization, and had no neutralizing antibody in their nasal secretions. All were infected with the virus. Two of the remainder, one with a high titre of circulating antibody and the other with a low titre, did have nasal antibody and neither was infected. Of the remaining two, one had a high titre of circulating antibody and no nasal antibody and was shown to be infected both by virus isolation and subsequent antibody rise; the other had an insignificant titre of circulating antibody and no nasal antibody and apparently could not be infected. The overriding influence of local antibody is amply indicated in this small series except in the case of the last volunteer, and it is possible that in his case the test was not sensitive enough to detect it.

DISCUSSION

The effects of the experimental live vaccine made from an influenza type B virus that was given repeatedly to a group of factory workers in the Midlands can be briefly summarized as follows: (1) a high rate of infection initially (Table 1), (2) considerable antibody formation in those infected (Table 1, Fig. 2), and (3) a high incidence of clinical reactions (especially after the first vaccination) which appeared to be a reflexion of residual pathogenicity in the virus (Table 4). All the effects were sharply reduced after the second vaccination and were almost wholly absent after the third (Tables 1–4). Most of the people without antibodies at the beginning of the trial developed them later, but about 8 % failed to do so and some of these actually excreted virus. Seven months after vaccination a challenge with the same virus showed that immunity had persisted, but there was a suggestion that it was then beginning to wane. Finally the vaccine gave promise of a protective effect against related serotypes (Table 7).

The difficulty of achieving infections and immunological responses with live influenza vaccines without clinical reactions has often been commented on. The high rate of recorded reactions in our trial (Table 5) could have been due in part to suggestion and to intercurrent infection, and evaluation of the results would have been easier if we had been able to vaccinate a control group with placebo. A much lower rate of clinical reactions was observed in the nine Salisbury volunteers and this may have been due to a better standard of clinical surveillance or to the different conditions that prevailed there. Methods of administering the virus may also have affected the clinical response: a spray which was used at Wolverhampton could certainly be expected to produce a greater and more widespread effect on susceptible cells than a simple dropper.

In the main it was the people without initial antibodies who were most readily infected (Tables 2, 4) and it was they who provided the bulk of antibody rises later (Table 3). In the former respect the results of this trial were at variance with those of the trial of live vaccine prepared from a Russian A_2 virus a few years before (McDonald *et al.* 1962). On that occasion it appeared that nearly all the volunteers with antibodies before vaccination were infected by the virus since they produced a boost of antibodies later. However, there were certain people in the present trial who were similarly proved to be infected in spite of their previous high antibody titres. Another curious feature of the earlier trial was that, although the volunteers without initial antibodies were readily infected and excreted virus for several days after vaccination, they formed no antibodies; nevertheless, when challenged with vaccine 1 month after the vaccination they were evidently immune, since the virus could not then be isolated. Thus low antibody titres did not denote susceptibility to infection at the time of revaccination. In the present trial, although antibodies were produced after the first vaccination, something of the same trend was seen in that a low level of antibody was compatible with protection against the second and third virus dose (Table 4). This feature was also exemplified in antibody rises; a quite small rise after the first vaccination was rarely followed by another rise after the other vaccinations (Table 3; Figs. 3–4).

Although the reason why some people failed to respond serologically to vaccination and the mechanism by which they were later protected is not precisely known, it seems clear that circulating antibody is only one factor in protection against influenza. Smith *et al.* (1966) found poor correlation between resistance to infection against parainfluenza type 1 virus and serum antibody. But it agreed well with the presence of nasal antibody. In our limited investigation of this aspect of the problem we found the same and it seems likely that it is the induction of local antibody by live influenza vaccines which makes them immunologically effective.

SUMMARY

A trial of an experimental live influenza B vaccine has been described.

The virus it contained was active and produced infections, antibody rises and clinical reactions.

Second and third vaccinations had much less effect than the first. Resistance to revaccination was only partially reflected in the serological response.

It seems that another factor, probably local antibody, exerts a considerable influence on resistance to infection with influenza viruses.

We are greatly indebted to Dr P. G. Higgins of the Public Health Laboratory, Cirencester, who went to much trouble to provide us with specimens from patients with influenza; to Dr H. G. Pereira of the National Institute for Medical Research for the antigenic analysis of the vaccine virus; to Messrs Sankey Ltd., Bilston, Wolverhampton, for their unfailing courtesy and forbearance throughout the trial; to the volunteers for their enthusiastic co-operation in the face of some discomforts; and to Messrs Pfizer Ltd., Sandwich, for originally providing facilities for the preparation of the vaccine.

In particular we wish to record our gratitude for the invaluable technical help of Miss Pamela Ball of the Common Cold Research Unit, Mrs Maria Gregory of the Bacteriology Department of Liverpool University, Mrs L. Johnson of the Virus Laboratory of New Cross Hospital, Wolverhampton, and Mr A. Westoby, an assistant in the practice of Dr Tyler.

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.

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 (A 2) strain. (A report to the Medical Research Council by their Committee on Influenza and Other Respiratory Virus Vaccines.) *J. Hyg., Camb.* **65**, 245.

COMMUNICABLE DISEASE CENTER (1965). *Influenza Surveillance Report*, no. 81, pp. 21–3.

HENLE, W. & LIEF, F. S. (1963). The broadening of antibody spectra following multiple exposures to influenza viruses. *Am. Rev. resp. Dis.* **88**, no. 3, ii, 379.

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.

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.

PEREIRA, M. S., CHAKRAVERTY, P., POLLOCK, T. M. & POPE, J. G. (1967). Survey of influenza antibody in England by the strain-specific complement-fixation test. *Br. med. J.* iv, 80.

SEVER, J. L. (1962). Application of a microtechnique to viral serological investigations. *J. Immun.* **88**, 320.

SMITH, C. B., PURCELL, R. H., BELLANTI, J. A. & CHANOCK, R. M. (1966). Protective effect of antibody to parainfluenza type 1 virus. *New Engl. J. Med.* **275**, 1145.

SMORODINTSEV, A. A., ALEXANDROVA, G. A., CHALKINA, O. M. & SELIVANOV, A. A. (1965). Experience in the development of live vaccines against influenza and influenza-like respiratory infections. *Appl. Virol.: 1st Annu. Symp., Boca Raton, Fla.*, 1964, p. 142.

WORLD HEALTH ORGANIZATION (1953). Expert Committee on Influenza. *Tech. Rep. Ser. Wld Hlth Org.* no. 64.

J. Hyg., Camb. (1969), **67**, 13
Printed in Great Britain

A new approach to development of live vaccine against tick-borne encephalitis

By A. A. SMORODINTSEV, A. V. DUBOV, V. I. ILYENKO and V. G. PLATONOV

Institute of Experimental Medicine, Academy of Medical Sciences U.S.S.R., State Research Institute of Influenza, Leningrad, U.S.S.R.

(*Received* 26 *February* 1968)

Tick-borne encephalitis (TBE) virus, like many other arboviruses, is very stable in genetic characteristics such as neurovirulence. Extreme difficulty has been encountered in efforts to reduce its pathogenicity artificially and therefore many attempts in the U.S.S.R. to attenuate the virus and to develop a live vaccine for mass vaccination have been unsuccessful. A killed formol-vaccine has therefore been widely used since 1939 (Smorodintsev, Levkovich & Dankovsky, 1940; Smorodintsev *et al.* 1941), at first made in mouse brain and later in tissue culture (Smorodintsev & Ilyenko, 1961). Unfortunately such vaccines have to be given annually and a more effective and convenient live vaccine would be a considerable advantage if safe and immunogenic vaccine strains could be found.

Our first step in this direction was the study of the Langat (TP 21 strain) virus (Smith, 1956) because of its close antigenic relationship to TBE and absence of known natural disease caused by it in humans in Malaya. The mouse-adapted variant of Langat virus can be sharply differentiated from typical TBE virus on the basis of its inability to infect mice by intraperitoneal (IP) or subcutaneous (SC) inoculation and rhesus monkeys by intracerebral (IC) injection. The original Malayan strain grows poorly in chick embryo cells (CEC) at 40° C. and does not grow at 41·5° C. The original mouse brain virus contains a mixture of strains more or less pathogenic for mice, monkeys and man. The proportion of more virulent virus in the original mouse brain virus is very small but increases very sharply after five or more passages in CEC. Using terminal dilutions (which cause less than 50 % mortality in mice infected IC) thirty-eight pure clones of Langat virus including two virulent and thirty-six avirulent variants have been isolated from mouse-brain virus. These two main types of variant were genetically stable and were not changed in virulence by prolonged passage at 36° or 40° C. in tissue culture or by prolonged mouse-brain passage.

Table 1 compares the immunogenic activity of a standard killed TBE vaccine with live vaccines produced from variants of Langat virus. After three doses of standard formol-vaccine, 124 (84 %) of 148 susceptible persons developed neutralizing antibodies: titres in 11 % were low, in 26 % medium and in 47 % high. Live tissue-culture vaccine from the avirulent Langat strain gave much poorer immune responses than the corresponding live brain vaccine (70 % success, 30 % negative or low titres). This probably depended on the differences in virus concentration

in the vaccines: 8·0 log LD 50/0·03 ml. in brain vaccine but only 5·5–6·5 log. LD 50/0·03 ml. in tissue culture vaccine. With only one dose of a live tissue culture vaccine, made from virulent Langat virus, the antibody responses were best of all.

Table 1. *Neutralizing antibody responses 1½ months after vaccination with killed tick-borne encephalitis or live Langat vaccines*

Vaccine	No. of doses	No. vaccinated	Neutralization test positive		Percentage with log. neutralization index			
			Total	%	Neg. 0–2·0	Low 2·1–3·0	Medium 3·1–5·0	High >5·0
Killed tissue culture vaccine from biphasic encephalitis virus	3	148	124	84	16	11	26	47
Live brain vaccine from avirulent Langat strain	2	76	67	88	12	22	20	46
Live tissue culture vaccine from avirulent Langat strain	2	66	46	70	30	45	25	0
Live tissue culture vaccine from virulent Langat strain	1	62	60	97	3	3	32	62

Table 2. *Changes in virus neutralizing antibody titres 1½ months and 3 years after immunization of volunteers by killed biphasic or live Langat vaccine*

Vaccine	No. of doses	Log. LD 50 per dose	No. vaccinated	Time after vaccination (months)	Number with log. neutralization index			
					Neg. 0–2·0	Low 2·1–3·0	Medium 3·1–5·0	High >5·0
Killed tissue culture vaccine from biphasic virus	3	9·0	24	1½	0	0	2	22
				36	17	6	1	0
Live brain vaccine from avirulent Langat virus	2	9·0	34	1½	0	10	9	15
				36	4	14	11	5
Live tissue culture vaccine from avirulent Langat virus	2	5·5–6·5	23	1½	—	8	15	0
				36	6	6	11	0

Table 2 shows the result of repeat tests 3 years after the immunization with killed and live avirulent vaccine, on those volunteers who had shown a distinct serological conversion 1½ months after vaccination. Volunteers vaccinated with killed vaccine showed a pronounced fall in antibody level, while those immunized with live brain or tissue culture vaccine showed more stable levels. In the first group (three doses killed vaccine) 17 (71 %) of 24 originally seropositive persons became negative; in the second group (two doses live brain vaccine) only 4 (12 %) of 34, and in the third group (two doses live tissue culture vaccine), only 6 (26 %) of 23 became negative. Thus immunity lasts longer after two doses of avirulent live vaccines than after three doses of formol-vaccine.

Studies of more than 1000 people vaccinated with the avirulent clone of Langat strain show that it is safe and does not provoke any general clinical or neurological reactions. Vaccination was not followed by viraemia except in a few subjects

during the first few days. Tissue culture vaccine from an avirulent variant of Langat strain is thus very promising as a safe and more potent substitute for killed formalinized vaccine.

However, as the immunizing activity of this avirulent variant of the Langat strain is limited, especially after one dose, a vaccine strain with higher immunogenic potency is desirable. Naturally attenuated strains were therefore sought among TBE viruses isolated directly in tissue culture from the ticks *Ixodes persulcatus* or *I. ricinus*. Such strains were isolated in West Siberia and Kirgiz Republic, where human disease is rare but infection of man by ticks is very intensive.

Pronounced differences are known between the severity of the disease and the mortality rate from the paralytic form of TBE found in the Far East, and the milder form of disease found in the European part of the U.S.S.R., as well as between the paralytic and biphasic forms of the disease observed in U.S.S.R. and other European countries.

Widely different biological variants of TBE virus can be isolated from infected ticks but not from the brains of patients. Among the tick strains a group has been found which are only slightly pathogenic for mice by SC inoculation and non-pathogenic for rhesus monkeys by IC inoculation. Strains of this type have not been isolated from clinical cases of TBE whether of the severe paralytic or the much more benign biphasic meningoencephalitis form.

So far seventy-seven strains have been studied of the tick-borne and biphasic meningoencephalitis viruses isolated from various sources in different geographical zones from the Far East to Austria, and eight strains non-pathogenic for rhesus monkeys by IC inoculation have been isolated in Western Siberia (Isetsk district of the Tyumen region), in Kirghizia (Kamenetak district), and in European U.S.S.R. (Novgorod and Vologodsk regions). All these latter areas have high infection rates in ticks but very little clinical tick-borne encephalitis—an average of 10 % of the individual larval ticks were infected. These non-pathogenic strains cause little or no disease in monkeys and complete recovery is the rule. Besides these, strains were also recovered which caused persistent brain infections in monkeys and which frequently provoked chronic or progressive disease similar to the rarely observed chronic form in human beings. These strains may provide a laboratory model in monkeys for the study of the pathogenesis of severe complications of TBE in man.

Strains from an area with a high infection rate but a low level of tick-borne encephalitis morbidity in the subzone of aspen and birch woods of Western Siberia (Tyumen region) were compared with TBE strains from foci with a high morbidity (Yashkin district of the Kemerovo region) of typical TBE.

MATERIALS AND METHODS

Thirteen strains from Western Siberia were selected for detailed study (all from ticks except one from blood of a healthy human being): 4 from Kemerovo (K 1-1 to K 1-4) and 1 from Novorsibirsk (N 1-1); and 8 from Tyumen (T 2-1 to T 2-5, T 3-1 to T 3-3). T 3-1 is the Elantsev strain (from human blood), T 3-2 the Isetsk-237 strain and T 3-3 the Isetsk-9 strain.

ห้องสมุด กรมวิทยาศาสตร์

These strains, which had been stored at −20° C. in mouse brain for not more than 2–3 days, were each inoculated into mice and CEF cultures. Their pathogenicity was studied in mice of 10–12 g. and in rhesus monkeys. Mouse brains were removed when symptoms appeared. For titrations mice were inoculated with volumes of 0·03 ml. IC and 0·25 ml. SC. Monkeys were inoculated IC with 0·5 ml. CEF tubes were inoculated with 3·0 log. LD 50/0·03 ml. virus, then incubated at 30°, 36° or 40° C. During 5 days, at intervals of 24 hr., 7–10 tubes were taken from each incubator and the pooled culture fluid titrated IC in mice.

RESULTS

As a result of these tests the strains could be classified into three pathogenicity groups: (I) strains with a high extraneural (SC) pathogenicity for mice and a high neurovirulence for monkeys; (II) strains with a high extraneural pathogenicity for mice, but with irregular monkey mortality, and (III) strains with a low extraneural virulence for mice and causing no clinical symptoms after IC inoculation of monkeys. In addition growth indices in CEF cultures at optimal (36°), above optimal (40°) and below optimal (30° C.) temperatures were used for classification.

Table 3. *The pathogenic activity of various strains of tick-borne encephalitis virus tested in mice*

		LD 50 ± S.E.			
		Intracerebral Inoculation		Subcutaneous inoculation	
Pathogenicity group	Strain	Freshly isolated	Mouse brain adapted	Freshly isolated	Mouse brain adapted
I	K 1-1	9·5 ± 0·2	9·4 ± 0·13	7·5 ± 0·32	7·5 ± 0·2
(Kemerovo)	K 1-2	8·8 ± 0·34	8·7 ± 0·19	5·0 ± 0·3	5·1 ± 0·2
	K 1-3	9·6 ± 0·23	9·6 ± 0·1	6·5 ± 0·39	6·6 ± 0·1
	K 1-4	9·5 ± 0·3	9·5 ± 0·12	6·0 ± 0·2	6·1 ± 0·2
	N 1-1	9·2 ± 0·3	9·3 ± 0·2	7·6 ± 0·35	7·6 ± 0·2
	Mean	8·3 ± 0·08	9·3 ± 0·08	6·5 ± 0·23	6·6 ± 0·2
II	T 2-1	6·1 ± 0·3	9·0 ± 0·12	4·2 ± 0·21	5·3 ± 0·1
(Tyumen)	T 2-2	6·3 ± 0·2	9·3 ± 0·9	6·6 ± 0·28	6·8 ± 0·19
	T 2-2(?)	7·0 ± 0·24	9·1 ± 0·11	4·3 ± 0·3	6·1 ± 0·19
	T 2-5	6·5 ± 0·21	8·9 ± 0·13	4·7 ± 0·3	5·9 ± 0·17
	Mean	6·9 ± 0·58	9·0 ± 0·04	4·9 ± 0·19	5·8 ± 0·15
III	T 3-1	8·6 ± 0·3	7·8 ± 0·08	2·0 ± 0·19	2·2 ± 0·25
(Tyumen)	T 3-2	8·0 ± 0·3	8·2 ± 0·1	2·2 ± 0·21	2·2 ± 0·27
	T 3-3	8·3 ± 0·25	8·5 ± 0·13	2·4 ± 0·22	2·3 ± 0·2
	Mean	8·2 ± 0·13	8·3 ± 0·1	2·2 ± 0·13	2·3 ± 0·16

The SC susceptibility of mice was one of the important and stable properties for differentiation (Table 3). After adaptation to mouse brain, viruses in groups I and II had not only a high IC but also high SC virulence. By these markers strains of groups I and II could not be differentiated either from one another or from the

most typical highly pathogenic strains isolated from the brains of paralysed patients in the Far East. The degree of SC virulence distinguished the strains in group III. After mouse adaptation group III strains still had a very stable low

Table 4. *Clinical symptoms in rhesus monkeys infected intracerebrally by viruses of different neurovirulence*

Pathogenicity group	Strain	The doses (log LD 50/ml.)	Clinical symptoms	Viraemia	Result
I	K 1-1	7·5	Paralysis of limbs	+	Death
	K 1-2	7·0	Paralysis of limbs	+	Death
	K 1-3	7·6	Paralysis of limbs	+	Death
	K 1-4	7·5	Paralysis of limbs	+	Death
	N 1-1	7·2	Paralysis of limbs	+	Death
II	T 2-1	8·4	Ataxia	+	Death
		9·7	Ataxia	±	Death
	T 2-2	7·5	Ataxia		Recovery
	T 2-3	9·7	Ataxia	±	Death
		8·0	Ataxia	+	Death
		5·0	Ataxia	+	Death
		8·5	Ataxia	+	Recovery
		3·0	Nil	+	No illness
	T 2-4	6·8	Ataxia, left hemiparesis		Death
		8·7	Ataxia, paraplegia	+	Recovery
	T 2-5	8·2	Ataxia, paraparesis left leg	+	Recovery
		8·5	Ataxia	+	Recovery
III	T 3-1	3·9	Nil	±	No illness
		6·9	Nil	–	No illness
		7·5	Nil	–	No illness
		7·7	Nil	±	No illness
		8·4	Nil	±	No illness
	T 3-2	3·5	Nil	±	No illness
		6·8	Nil	±	No illness
		7·7	Nil	–	No illness
		8·4	Nil	–	No illness

Table 5. *Final titres in primary chick embryo cultures at* 40°, 30° *and* 36° C.

Pathogenicity group	Strain	40° C.	30° C.	36° C.
I	K 1-1	5·8 ± 0·51	5·9 ± 0·42	5·9 ± 0·49
	K 1-2	5·7 ± 0·52	5·6 ± 0·43	5·9 ± 0·46
	K 1-3	6·2 ± 0·4	5·3 ± 0·41	6·2 ± 0·39
	K 1-4	6·2 ± 0·37	6·0 ± 0·42	6·3 ± 0·58
	N 1-1	5·9 ± 0·4	5·9 ± 0·46	5·9 ± 0·72
	Mean	5·9 ± 0·2	5·8 ± 0·21	6·0 ± 0·23
III	T 3-1	0·7 ± 0·29	4·5 ± 0·25	4·5 ± 0·43
	T 3-2	0·04 ± 0·003	5·0 ± 0·5	4·4 ± 0·21
	T 3-3	0·2 ± 0·09	3·5 ± 0·33	3·3 ± 0·36
	Mean	0·3 ± 0·09	4·3 ± 0·24	4·1 ± 0·24

SC virulence for mice. The difference between the IC LD 50 and SC LD 50 was more than 5·0 log.

A still more important marker for the differentiation of the tick-borne encephalitis virus into three different biological groups was provided by IC inoculation of monkeys (Table 4). Group I strains did not differ from 'typical' highly pathogenic strains and caused atrophic paralysis with an intense and regular viraemia lasting until death in all, in 7 days or less. Group II strains characteristically caused cerebellar symptoms in the infected monkeys (ataxia, in single cases an intention tremor and nystagmus). Some of the group II strains (T 2-4, T 2-5) also caused limb paralysis. Most monkeys infected with group II viruses also had a stable viraemia lasting 7–10 days. However, group II strains caused death of only about half of the infected animals, the rest recovering completely or with sequelae.

In monkeys infected with large amounts (6·8–8·4 log. LD 50) of group III strains no noticeable pathological changes were found in the nervous system. The only reaction was a short-term temperature rise. In many cases viraemia was absent.

Table 6. *Classification and basic properties of the various strains of tick-borne encephalitis virus isolated in West Siberia*

Pathogenicity group	Strain	Subcutaneous mouse LD 50	Intracerebral susceptibility of monkeys: Clinical	Viraemia	Results	Maximum titre on the chick-embryo cultures at 40°C.
I (Kemerovo)	K 1-1	7·5 ± 0·23	Paralysis	+	Death	5·8 ± 0·51
	K 1-2	5·1 ± 0·29	Paralysis	+	Death	5·7 ± 0·52
	K 1-3	6·6 ± 0·14	Paralysis	+	Death	6·2 ± 0·4
	N 1-1	7·6 ± 0·25	Paralysis	+	Death	5·9 ± 0·4
	Mean	6·6 ± 0·22	Paralysis	+	Death	5·9 ± 0·2
II (Tyumen)	T 2-1	5·3 ± 0·14	Ataxia	±	Death irreg.	3·5 ± 0·27
	T 2-2	6·8 ± 0·19	Ataxia	±	Death irreg.	2·6 ± 0·06
	T 2-3	5·2 ± 0·14	Ataxia	+	Death irreg.	3·1 ± 0·08
	T 2-4	6·1 ± 0·19	Ataxia paresis	+	Death irreg.	2·7 ± 0·46
	T 2-5	5·9 ± 0·17	Ataxia paresis	±	Death irreg.	3·1 ± 0·2
	Mean	5·8 ± 0·15	Ataxia	+	Death irreg.	2·9 ± 0·2
III (Tyumen)	T 3-1	2·2 ± 0·25	No illness	–	No illness	0·4 ± 0·29
	T 3-2	2·2 ± 0·27	No illness	–	No illness	0·04 ± 0·003
	T 3-3	2·3 ± 0·2	No illness	–	No illness	0·2 ± 0·09
	Mean	2·3 ± 0·16	No illness	–	No illness	0·3 ± 0·09

In CEF cultures (Table 5), all the group I strains grew at 40° C. more intensively (average titres 5·9 ± 0·2 log. LD 50/0·03 ml.) than the strains of group III. Group III strains differed essentially in that they either failed to multiply or grew very poorly (average titre 0·3 ± 0·09 log. LD 50/0·03 ml.) (Table 6). At 30° and 36° C., group I strains multiplied as actively as at 40° while group II strains multiplied better at 30° and 36° than at 40° C.

The correlation between neuro-virulence in mice and monkeys with the ability

of the same strains to grow at 40° C. indicates the importance of growth at this temperature as a genetic marker of virulence for susceptible animals. The neurovirulence marker of the TBE virus seemed to be related to the ability to multiply under unfavourable conditions of raised temperature.

SUMMARY

Investigations of the biological properties of tick-borne encephalitis viruses immediately after isolation from naturally infected *Ixodes persulcatus* from Western Siberia has revealed their different pathogenicity for monkeys and mice. Strains highly pathogenic for mice on extraneural inoculation and for rhesus monkeys on intracerebral inoculation have been isolated, but also strains with a low extraneural pathogenicity for mice and a complete non-pathogenicity or a mild pathogenicity for monkeys on intracerebral inoculation.

The viruses have been classified into three main groups by their virulence for mice and monkeys, and their ability to grow in tissue culture at 40° C. and the findings are summarized in Table 6. Group I included viruses highly virulent for mice by any route of inoculation, which caused fatal paralytic disease in monkeys, and which grew well in chick embryo culture at 40° C.

Group II strains also had a high virulence for mice, but in monkeys caused milder disease with a peculiar clinical course indicative of primary damage to the cerebellum. About half of the infected monkeys recovered completely or with mild sequelae (ataxia, pareses of the limbs). This group grew moderately well in chick embryo cultures at 40° C. Groups I and III differed essentially in their virulence for mice by subcutaneous inoculation, and virulence for monkeys decreased distinctly with transition from group I to group III. Group III strains showed a lower reproduction rate at 40° C. in chick embryo culture than group I. Groups I and II showed good correlation between extraneural pathogenicity for mice, pathogenicity for monkeys by IC inoculation, and growth rate in chick embryo cultures at 40° C.

Group I strains were isolated chiefly from foci with a high TBE morbidity and the strains of groups II and III in foci with sporadic cases. It is, however, highly probable that in any tick-borne encephalitis focus several variant strains of differing virulence exist.

Recent experience of vaccinating volunteers with naturally attenuated group III strains suggests that safe and effective live vaccines can be developed against tick-borne encephalitis. Vaccines against many other arbovirus diseases can probably also be derived from naturally occurring strains of virus isolated from arthropods in areas of low disease but high infection rate. This approach looks more promising than the usual approach of attempting to develop attenuated strains from highly virulent strains.

REFERENCES

Smith, C. E. G. (1956). A virus resembling Russian spring–summer encephalitis virus from an ixodid tick in Malaya. *Nature, Lond.* **178**, 581–2.

Smorodintsev, A. A. & Ilyenko, V. I. (1961). Results of laboratory and epidemiological study of vaccination against tick-borne encephalitis. *Proc. Symp.: Biology of Viruses of the Tick-borne Encephalitis Complex* (ed. H. Libikova), pp. 332–43.

Smorodintsev, A. A., Kagan, N. W., Levkovitch, E. N. & Dankovsky, N. L. (1941). Experimenteller und epidemiologischer Beitrag zur activen Immunisierung gegen die Frühling-Sommer-Zechen-Encephalitis. *Arch. ges. Virusforsch.* **2**, 1–25.

Smorodintsev, A. A., Levkovitch, E. N., & Dankovsky, N. L. (1940). Experience in preventing spring–summer encephalitis in the endemic areas with killed virus vaccine. *Arch. biol. Sci.* **59**, 92–104. (In Russian.)

J. Hyg., Camb. (1969), **67**, 21
Printed in Great Britain

The outbreak of foot-and-mouth disease in Worcestershire

An epidemiological study: with special reference to spread of the disease by wind-carriage of the virus

BY R. J. HENDERSON
Public Health Laboratory, Worcester

(*Received* 15 *May* 1968)

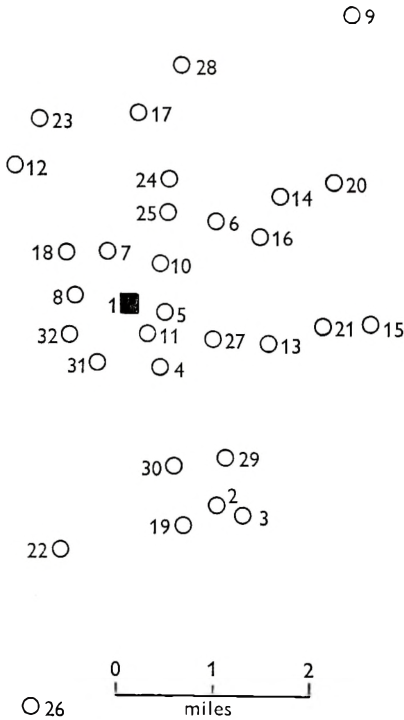
During the recent epizootic of foot-and-mouth disease which attacked the country in the winter of 1967/8, Worcestershire was among the affected counties. The area affected is known as Spetchley. The disease appeared in three pig farms on 16, 17 and 18 November 1967 respectively. These farms are owned jointly by two brothers. It is understood that the disease travelled to Worcestershire in a tanker load of skimmed milk and some of the load was distributed equally among the three pig farms. The disease spread out radially from the first of the three pig farms and later from the second until twenty-nine neighbouring farms were involved, and only died out with the infection of the thirty-second farm on 6 December.

On a visit to the control centre at Hindlip when the Worcestershire outbreak was nearing its end I was shown a plan which had been prepared by Mr J. S. Needham of the control staff. On it Mr Needham had endeavoured to show that the spread of the infection from the source to the neighbouring farms could, in most cases, have been by wind. The impression of the farmers as repeated to me by Mr Needham seemed to confirm this. I thought that the matter of wind carriage should be looked at closely.

When the outbreak was over therefore and one was free to move about, I paid a visit to each of the farms which had been attacked, learned as much as I could from the farmer himself about the arrival of the disease, discussed the precautions he had taken, and so on. I thought at the time that one or two farmers after a lapse of 8 weeks might perhaps have forgotten the sequence of events. I need not have worried. Each farmer appeared to be absolutely clear about the timing of events, remembering even the smallest detail. The boundaries of each farm were marked on a 2½ in. to the mile map; inquiries were made about the exact disposition of the animals at the time when I judged the infection had arrived on the farm, and about the direction in which the sheds, barns, and byres faced. When all this was done, I visited the R.A.F. Meteorological Station at Pershore (only a few miles from the outbreak) where hourly records of wind force, wind direction, rainfall, and other climatic conditions are kept. I received the information I required and my conclusions are in the following pages.

THE PART POSSIBLY PLAYED BY THE WIND

The infection was first noticed at one of the pig farms (black square on Fig. 1) on 15 November (lameness and raised temperature in two sows and two piglets). The diagnosis was confirmed on 16 November and two more piglets were then seen to be infected. The infected animals were slaughtered on 16 November and the remaining stock slaughtered and buried by 21.00 hr. on 17 November. Maximum spread of infection from this farm was likely therefore on 15, 16 and 17 November. Was it possible that the infection was spread to neighbouring farms on the wind? It seems that this could happen in either of two ways. The first is as follows.



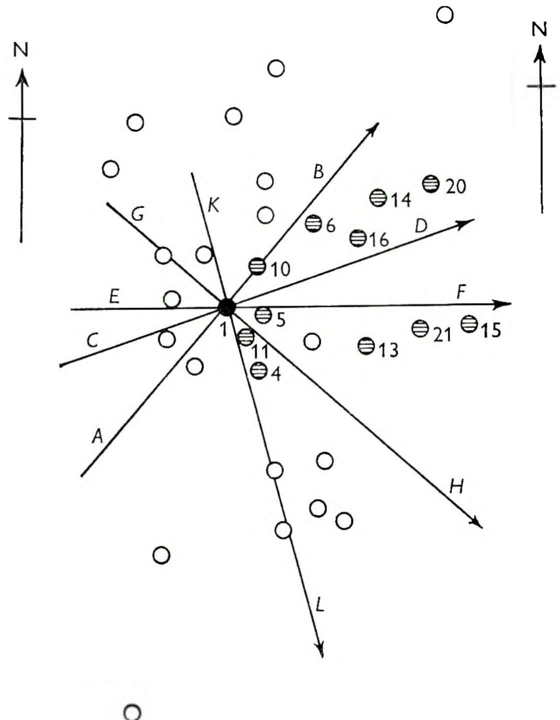


Fig. 1 Fig. 2

Fig. 1. The farms affected in the Worcestershire outbreak. The numbers indicate the order in which infection appeared. Farm 9, 16 miles distant at Kidderminster, was infected by means other than by the wind and is not considered further in the text.

Fig. 2. Farms possibly infected on 15. xi. 67. from farm 1 (focus of infection). Farm 1 is shaded black. The infected farms are shown by cross-hatching.

Virus particles are expelled into the air on the breath or saliva of infected animals or from the animals' chests which are squeezed when the carcases are dragged by the tractors, or from blisters ruptured when the dragging chains are applied, or attached to dust particles when the excavators dig the burial pits; the wind could lift the virus particles and carry them along with it. Some of the particles could be brushed off on any obstacle in the path of the wind (haystacks, dutch barns, sheds, trees, cattle); some on to crops such as kale or grass, particularly if they are wet.

There is every possibility that this happened, and the accompanying diagrams show the wind direction throughout the Worcestershire episode.

On 15 November (Fig. 2) a light breeze (1–3 knots)* came from the south-west virtually all day, veering between lines *AB* and *CD*. At 21.00 hr. it changed to a westerly direction along line *EF* (strength about 10 knots). Then from 21.00 to 24.00 hr. it blew from the north-west between lines *GH* and *KL* (strength slightly over 10 knots). Thus farms lying in the path of the wind were in danger of being infected about the same time on the 15th. As it so happened farms down-wind

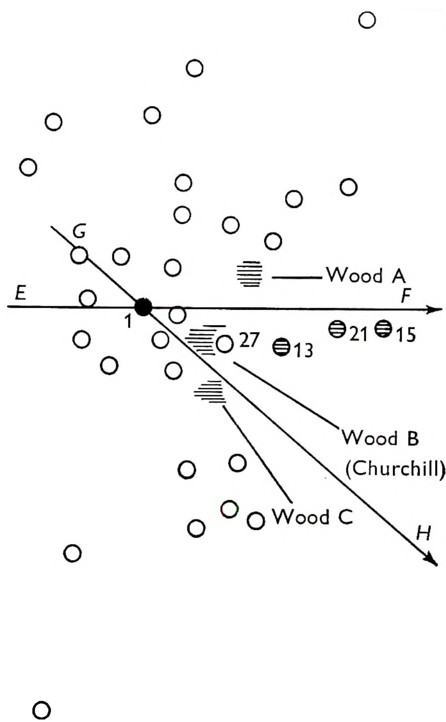


Fig. 3

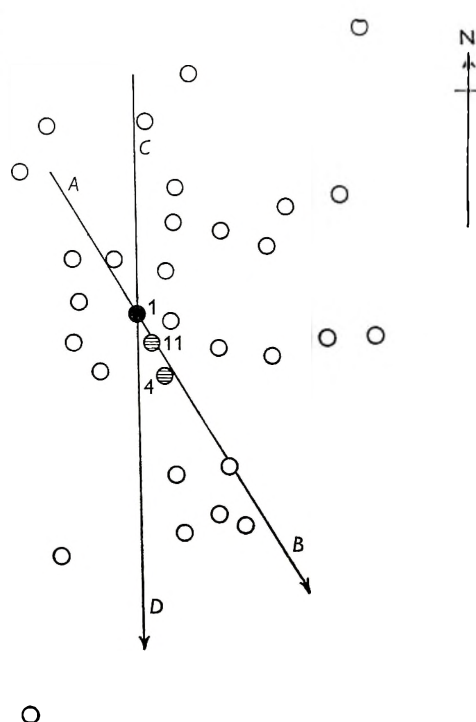


Fig. 4

Fig. 3. Protection of farm 27 by Churchill Wood. Farms 13, 15, 21, unprotected, became infected from farm 1.

Fig. 4. Wind direction on 16. xi. 67. Farms 4 and 11 infected from 1.

from the focus of infection and lying in paths roughly half a mile wide running north-east, east and south-east, were infected. Their position is indicated in Fig. 2. Disease broke out on these farms between 20 and 25 November.† The farms nearest the focus showed the disease first, those further away showed it later. There was one notable exception. Considerable interest arose because one farm which lay in the path of the wind was not infected at the time with the others although it was one of those nearest the focus (Fig. 3). It is screened on its west and north flanks

* Beaufort scale equivalent given in Appendix I.

† A possible explanation of the apparent lengthening of the incubation period is given in the Discussion.

by a belt of trees, Churchill Wood, which is at least 300 yards wide. The trees are 40–50 ft. high. The assumption was that infection did not reach the farm buildings (the cows had been brought in immediately word was received of the outbreak) because they lay close in under the lee of the trees. The farm was actually infected much later when the wind was blowing in the reverse direction and it was presumed infection was carried to it from the neighbouring farm to the east (Fig. 7).

On 16 November the virus was probably being dispersed in greatest quantity from farm 1. A light breeze came from the north-west and north (strength

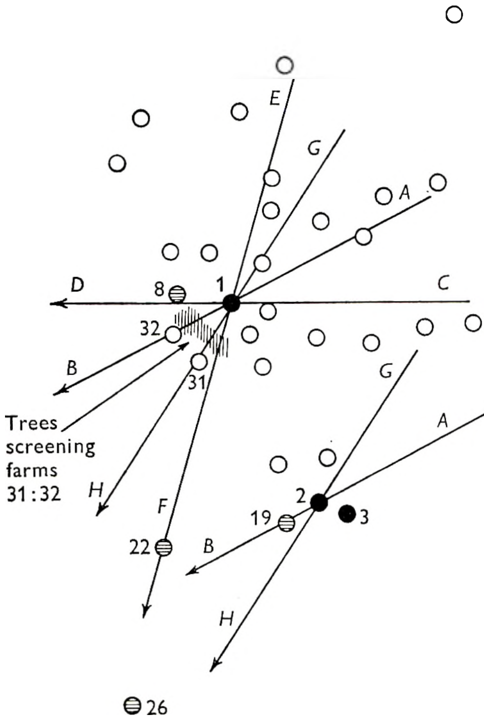


Fig. 5

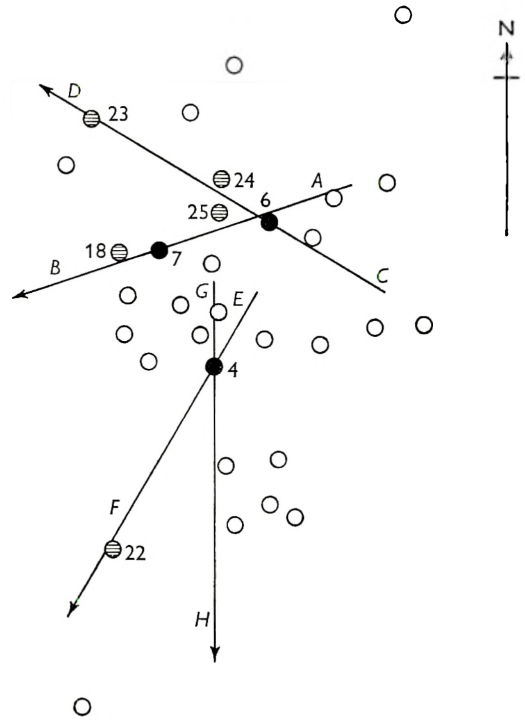


Fig. 6

Fig. 5. Wind direction on 17. xi. 67, 18. xi. 67 and 19. xi. 67. Farm 8 infected from 1, farm 19 infected from 2, farm 22 infected from 1, farm 26 infected from 2, farms 32 and 31 protected by trees.

Fig. 6. Wind direction on 20. xi. 67, 21. xi. 67 and 22. xi. 67. Farm 22 infected from 4, farm 18 infected from 7, farm 24 infected from 6, farm 25 probably infected from 6, farm 23 also infected from 6.

1–6 knots) along a line *AB–CD* from 00.01 to 17.00 hr. (Fig. 4). From then to 24.00 hr. it was completely calm. The farms lying in the path of the wind were subjected to a second day's attack by virus particles.

On 17 November the morning was calm from 00.01 to 11.00 hr. From 11.00 to 17.00 hr. a light breeze (strength 1–6 knots) blew along lines *AB–CD* (Fig. 5). From 17.00 to 20.00 hr. the breeze lay in the north-east (line *EF–GH*: strength 1–6 knots). In the calm periods on both 16 and 17 November a heavy dew fell. The disease broke out on two farms (8 and 22) on 22 and 25 November respectively.

It seems that farms 31 and 32, lying directly in the path of the wind *AB–GH*, ought to have been infected from farm 1, less than 1 mile away. It is likely that they were protected, as in the case of farm 27, by trees (Fig. 5). There is a thick belt of tall trees on both sides of Spetchley road giving ample protection from the north. Farms 31 and 32 were infected later from the south. There are no trees on their southern aspects.

A second source of infection now appears. The disease broke out on the second of the three pig farms (Farm 2) on 17 November (Fig. 5). The farm immediately adjacent to the west, 19, could have been infected on this or the following day. On the pig farm where the disease had originally broken out all the stock had been slaughtered and buried by nightfall on 17 November and total disinfection of the premises had begun.

On 18 and 19 November a light breeze came mainly from the north-east (line *GH*). The third of the three pig farms (farm 3) showed infection on the 18th.

The situation on 20, 21 and 22 November is shown in Fig. 6. A change now appeared because farms infected from the original focus began to show infection themselves and virus was dispersed from these new sources. From soon after 24.00 until 11.00 hr. on the 20th a light breeze blew (strength 1–6 knots) along line *AB*. Until 20.00 hr. it came along *CD*. From 20.00 to 24.00 hr. it was along line *EF* and at 24.00 hr. it backed to along line *GH*. On the 21st the breeze blew all day along line *EF–AB*. On the 22nd there was little change. Farm 18 was probably infected from 7; farm 22 from 4; farms 24 and 25 from 6.

On the 23rd a light breeze came from south-east and south along lines *AB–CD*, *EF–GH*. (Fig. 7). Farm 23 was probably infected from farm 6 and farms 24 and 25 subjected to a second day's attack from 6. The cattle were inside in farms 23 and 24 and this fact may have resulted in some slight delay in the infection reaching them. Farm 27, originally protected by the trees on its west flank, was now possibly infected from the farm immediately to the east. Its east flank was open and unprotected. The fact of the cattle being inside also offered no protection.

From 24 November to 2 December the wind came almost continuously from the south with slight variations to the south-east or to the south-west (Fig. 8). Five farms were probably infected now. One of these, 31, (Figs. 1, 8) was probably subjected to a constant barrage of virus particles from farm 22 immediately to the south of it since farm 22 was a source of heavy infection for various reasons* from 25 to 29 November. Farm 31, in addition to pedigree cattle, held a valuable herd of some 300 deer. These were watched daily by veterinarians with binoculars. One lame animal was penned and slaughtered for a check. It was uninfected. The herd of deer escaped† but the cattle on the farm showed heavy infection on 1 and 2 December.

* The veterinarians thought that the disease had been present for 48 hr. before it was reported, the infection was so advanced and widespread. In addition the slaughterers stopped work for a whole day because of some disagreement.

† There were many herds of deer exposed to infection in various parts of the country. Surveillance was thorough and not one animal was found to have caught the disease. I am informed that samples of blood did not show antibody formation. It can be assumed therefore that subclinical infection did not take place and the answer is simply that deer in this country are not susceptible.

Farms 30 and 29 were probably infected from farm 19. The owner of farm 30 was standing on raised ground listening to the sound of the excavators digging the burial pits on farm 19; the wind, he said, was blowing from the south, directly from farm 19 to his. He went in and told his wife that nothing now could save his stock. Six days later infection broke out among his sheep.

The last farm to be infected, 32 (Figs. 1, 8), could have received the virus from the neighbouring farm on 1 or 2 December. The disease showed itself on 6 December.

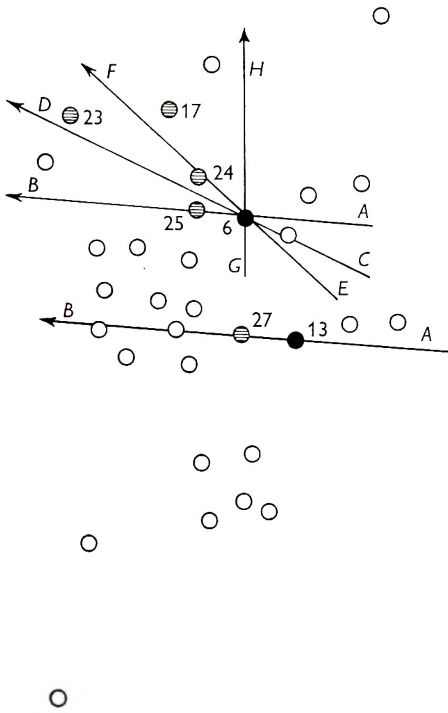


Fig. 7

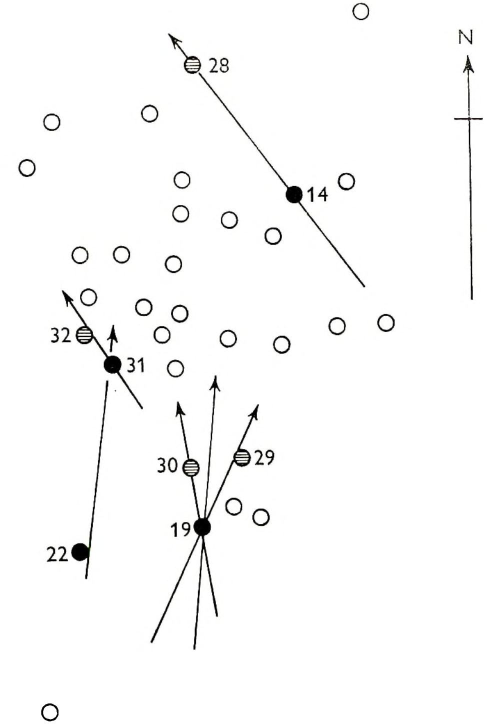


Fig. 8

Fig. 7. Wind direction on 23. xi. 67. Farm 27, hitherto protected by Churchill Wood from the west and south-west, is now infected by an east wind from farm 13. Farms 24 and 25 still infected from 6, farm 23 infected from 6, farm 17 also infected from 6.

Fig. 8. Wind direction between 24. xi. 67 and 2. xii. 67. Farms 29 and 30 infected from 19, farm 28 infected from 14, farm 31 infected from 22, farm 32 infected from 31.

The second way in which virus particles may be carried is rather more complicated and the effect of turbulence must be considered. Even in a gentle breeze (1–6 knots) there is an area of turbulence extending upwards from ground level to as high as 500 ft. Thus as virus is being dispersed the particles may be eddying up and down on the waves of turbulence while at the same time the whole mass moves along in the direction of the wind. While some particles in the lower reaches of the turbulence will be brushed off on features on the ground, the majority will remain aloft until brought down by light rain or settling of the dust. The particles themselves will fall during a period of complete calm.

Thus a plume down-wind of the original focus and perhaps extended for a distance of up to 3 miles could have been created by the breeze and turbulence on 15 and 16 November. From 17.00 hr. on the 16th until 11.00 on the 17th there was complete calm, with slight fog and a heavy dew. If it is believed that virus particles would be deposited during the period of calm then virtually all the farms to the east of the infected focus would have been in an area of fairly heavy concentration of virus. When the wind changed on 17 November and lay in the north-east and east the plume would tend to move west and west-south-west. When secondary outbreaks took place on the farms infected from the original focus the concurrence of light breezes, turbulence and calm would complete infection of the whole area.

DISCUSSION

For a long time there has been a feeling among veterinarians in this country that the virus of foot-and-mouth disease can be carried on the wind; in fact, on occasions the circumstantial evidence has been so strong that they have been obliged to accept this as the only way in which the disease could have spread. During outbreaks they have always advised farmers to get the stock under cover. Saliva from infected animals has been observed to be carried off by the wind and it has been proved experimentally that for a short distance at any rate the virus of foot-and-mouth disease can be carried through the air (Gowers Report, para 23). In this outbreak the phenomenon of saliva being blown off into the air was actually observed and the veterinarian who first saw it summoned his colleagues from the control point to watch. The animal was a young bullock which had been left out in a field on farm 29 (Fig. 1) near a road. From the road it was quite easy to see the wisps of saliva being borne off in the breeze for some distance.

However, when discussing the possibility of wind-borne spread of virus various climatic factors have to be considered. These are sunlight, rain, and calm. If virus is carried up by the wind, turbulence may take it to a considerable height; at the same time particles carried along near the ground may be brushed off on obstacles in the path of the wind. If there is strong sunlight or if it is a bright day the ultra-violet light will kill the virus in a matter of hours if not in minutes. A good deal will depend on how much mucus surrounds the virus or how much dust is attached to it. Therefore if the weather is bright and sunny much virus will not be carried along on the wind. However, if the wind is very strong or gusty some virus may be carried for considerable distances before the sunlight has had time to kill it.

The next factor is rain. This may have two effects. If the rain is light then it will help to bring the virus down out of the atmosphere and so on buildings, cowsheds, haystacks, fields and animals. Rain does no harm to a virus. If the rain is heavy, however, and if there is a lasting downpour then it is likely that all the virus in the air will be washed down into the ground or be carried away in ditches and gutters.

The third factor is calm. When the air is quite still virus particles surrounded by mucus or attached to dust may descend by their own weight.

Therefore when deliberating whether virus could have spread from the original focus, farm 1, to the surrounding farms, not only must the distance at which the

farms lie be taken into account but also any obstacle in the path of the wind, such as hills and woods. The direction and strength of the wind in the late afternoons, evenings and nights, the amount and type of rainfall during the period under review and the occurrence of any calm periods must be considered.

The incubation period also is important. This appeared to have been 5–6 days judging by the time the first signs of the disease took to appear on farm 1 after the arrival of the infected milk. In fact, veterinary officers agreed that the incubation period throughout the country was 5–6 days, and this was also the incubation period for the disease on those farms immediately adjacent to farm 1.

When spread of virus would have been at its maximum from the original focus (farm 1, Fig. 1), i.e. on the 15th, 16th and 17th, the prevailing wind for the first two of these days was from the west and on the third day from the north-west. On the 15th there was very little sunshine (0·8 hr. in fact); on the 16th 3 hr., on the 17th none. As regards rainfall, on the 15th there was very little (2·1 mm), on the 16th there was even less, and on the 17th none at all. Conditions therefore were suitable for the carriage of virus on the 15th; the very light rain would have helped to bring it down. On the 16th, however, the 3 hr. of sunshine might have killed some of the virus, although with the November sun not more than 20° above the horizon at noon the absorption of ultraviolet light by the atmosphere must have been considerable, and the effect of the light on the virus might therefore have been negligible. On the 17th virus would have survived in the air, but there was no rain to bring it down. There was a breeze on the night of the 15th and from 17.00 hr. on the 16th until 11.00 hr. on the morning of the 17th there was a complete calm. The wind strength throughout these 3 days apart from the period of calm was light to moderate (1–10 knots), i.e. a breeze sufficiently strong to have been felt on the face and at times strong enough to lift and carry dust. Atmospheric conditions therefore were suitable for the wind-borne spread of virus.

As it happened, farms to the north-east, and south-east were affected. Two farms showed the disease on the 20th, indicating an incubation period of 5 days if infection took place on the 15th, two on the 21st giving an incubation of 6 or 5 days depending on whether they were infected on the 15th or 16th, and the remainder, mostly the more distant farms, showed the disease on the 23rd, 24th and 25th. Why was the onset of the disease delayed on the more distant farms? The answer may lie in differences in concentration of virus. The concentration of virus over the distant farms was probably very light and this simply delayed contact with it. Another explanation may be that on farm 21 all the cattle were inside and the virus took longer to reach them, while on farms 13 and 15 most of the animals were inside and the remainder widely separated in the fields. This, together with the light concentration, would also tend to delay contact with the virus. As regards farm 22 where the incubation period appeared to be 7–8 days the veterinary officers consider that because the infection was widespread and heavy when confirmed it could have been present for 48 hr. before being reported. The limit of infection was about 3 miles. Farms beyond this were not affected.

On the diagrams the radial direction of spread following the path of the wind on

the 15th, 16th and 17th is striking. It is difficult to avoid the impression that virus was carried on the prevailing winds.

The theory of wind spread is also supported by what happened to farm 27 (Figs. 2, 3). This farm lay in the direct path of the wind on the 15th but it escaped infection, although farms to the east, west and south were infected. It seems that it was protected on its west flank by Churchill Wood, a thick belt of tall trees (40/50 ft.) 300 yards wide. The ground rises slightly here, bringing the tops of the trees to approximately 100 ft. above the level of farm 1. Thus either the virus was caught in the tree belt or, if turbulence was taking the virus up over trees, the farm buildings were protected from subsequent fall-out of virus by being under the lee of the trees. At any rate the farm escaped infection at this time. So did other farms to the north-east and south-east which were possibly protected by wood A and wood C (Fig. 3). It will be obvious how the westerly breeze would have been able to pass between woods A and B and so infect farms 13, 21 and 15. (Figs. 2, 3).

As it so happened, the disease broke out on farm 27 on 30 November 6 days after it was confirmed in cattle of farm 13. On the 23rd and 24th the wind came from the east, which explains how farm 27 could have been infected from farm 13 (Fig. 7). Thus there is strong circumstantial evidence that the farms to the north-east, east and south-east of farm 1 were infected by wind-carriage of virus in the early part of the Spetchley outbreak.

The remainder of the outbreak may be split into four periods: from 17 to 19 November; from 20 to 22 November; 23 November; and from 24 November to 2 December. As infection appeared on other farms, each in turn became a focus from which further spread was possible. It is true that there was a good deal of sunshine. However, on the days that the sun shone the wind lay in the same direction during the previous night and the same evening and night. It is also true that the dry spell continued for the most part, but slight amounts of rain fell on 25 November and from the 27th to the 30th. However, there were sufficient periods of calm to have allowed for deposition of virus.

Two farms, nos. 7 and 12 (Fig. 1) showed the disease on the 20th and 23rd respectively. These lay to the north-west of the original focus and direct infection by wind carriage cannot be postulated if a 5- or 6-day incubation period is accepted since the wind did not lie in this direction during the required time. It is not known how these farms became infected; a possible explanation is by westward movement of a plume (p. 27).

An interesting feature that emerged from the study was that when cattle had been brought inside they did not seem to be any better protected than those that remained outside. All that happened was that the spread of infection was delayed by 1 or 2 days. In nine farms the cattle had been brought inside, well before the outbreak began in two, and immediately on receiving word that the outbreak had begun in seven. In nineteen farms the animals (cattle or sheep) remained outside during the outbreak. The only difference between the two groups lay in the fact that infection took longer to reach those inside.

Although one farmer expeditiously brought his cattle into their sheds within an hour or so of hearing about the outbreak on farm 1 he continued to feed them on

cut kale from the field in which they had been grazing. The kale crop was in the direct path of the wind and he obviously could not have chosen a surer way of infecting his cattle with the virus.

The position of sheds and byres or the way in which they faced seemed to matter little. Although in some instances the doors of the sheds faced away from the prevailing wind there were ventilation openings in the shed walls. Short of hermetically sealing cow sheds or pig pens it is difficult to see how bringing cattle or pigs inside can do more than delay the onset of infection. This is especially so when bringing them in means that they have to be fed with hay which is likely to have been stored in Dutch barns. Dutch barns offer little protection against virus carried on the wind.

It seems feasible to make deductions about the range of carriage of the virus on the wind. On the 15th, 16th and 17th when spread from farm 1 was at its height, farms beyond 3 miles from the focus were not affected. In fact at no time during the outbreak was any farm which lay more than 3 miles from any focus affected. Since the wind was never stronger than 10 knots at any time during the outbreak it seems reasonable to suppose that a 10-knot wind will carry the virus up to 3 miles. Further, infection seemed solid on farms up to 1 or $1\frac{1}{2}$ miles distant from any focus, and less so, i.e. the virus was sparsely deposited, on farms at a distance of $1\frac{1}{2}$–3 miles. I postulate this because the disease took longer to appear on the more distant farms and it is possible that because the virus was sparsely distributed it took a little longer for the stock to come in contact with it.

This, if it is a correct interpretation, might be a useful guide to veterinarians during the course of future outbreaks. It is for consideration whether it would be worth while, when faced with a down-wind spread, ordering slaughter on all farms in the path of the wind for a distance far enough to ensure that the disease is stamped out quickly and completely.

Other possible methods of spread

Animals

Spread by foxes and other vermin was considered and discussed at length with farmers. A fox can pick up virus on his paws and coat when running through infected kale or grass. He might then infect other fields, but it is against his habits to enter cowsheds or pigpens. The farmers told me about any fox-runs and fox-hides on their land; on the whole they agreed that it would have been a very unusual coincidence if a fox or foxes could have accounted for all the disease in the Spetchley area. The manager of the pig farm where it all began had not seen a fox cross his fields before the outbreak or enter his pig pens. Other vermin, e.g. rats, were not thought by the farmers to be implicated. Rats usually remain close to a farm and do not leave. The only time rats were seen to move about was when farms were being dis-infected; there was no evidence however of their migrating to other farms.

Birds

Several farmers mentioned sparrows; it seemed they were numerous on some farms. Farmers agreed that sparrows remained near one farm and did not tend to move about in flocks from farm to farm. Only one farmer mentioned starlings. There was a flock which roosted at night in a wood near his farm and fed in his fields during the day. He did not think they would have gone to other farms although he had no proof of this. Pigeons were not thought to be implicated in any way since they did not come into pig pens or cowsheds. Pheasants, on the other hand, were blamed and one farmer described how when he was going round his fields he came on a bullock which obviously had the disease, the saliva dripping from its mouth having made a pool on the ground. As he approached, two pheasants got up which had, he said, been feeding on the saliva. Several farmers mentioned the possibility of the disease having been spread by pheasants; the Spetchley area is noted for them. However, in my view spread by these birds would have resulted in a very haphazard distribution not at all like that which was actually observed.

Persons

From the earliest days of the outbreak farmers in the neighbourhood of the affected farm observed the strictest precautions to prevent the disease from being brought to their farms by persons. Access gates were closed except one and a disinfectant 'pad' was laid across the entrance. Vehicles were not allowed to enter; domestic supplies and mail were taken at the gate; the farmer and his wife did not go out. If they were obliged to go out they disinfected their car. The children were kept at home. Workers on the farms disinfected their boots and changed their clothes where possible before going home in the evening. As more farms became infected the precautions became more strict. I went into the possibility of the virus being carried on persons or cars very thoroughly and I could find no common factor. It did not seem that transfer of virus on persons was playing any part in the outbreak.

Transfer by vehicles

The milk was collected from a large number of farms in the area by tanker. At one stage it was thought that the tankers were possibly transferring the disease from farm to farm. Investigation of the routes the tankers followed showed that this was not possible. In each case the route was the reverse of that taken by the disease. However, some farmers remained suspicious of the tankers, especially of the vacuum artificially created before taking in the milk. Some farms took in loads of animal feedingstuffs during the outbreak, but this was virtually always supplied in bags and had come from a source outside the infected area. Although two farms in another part of Worcestershire, unconnected with Spetchley, were infected from a haulier's lorry I did not find any evidence that carriage of virus on vehicles or on vehicle wheels played any part in the Spetchley outbreak; it seems a pity, however that 'progress' has done away with the safeguard of leaving the churns on a platform at the farm gate for collection (Gowers Report, para 170).

Transfer by veterinarians

In the Gowers Report, app. V, approximately ten instances of the 1951–2 outbreak where infection could have been brought to farms by veterinarians are described. Farmers had mixed views about allowing veterinarians to come into their premises to inspect stock; one whose farm escaped although there was infection virtually all around flatly refused to allow a young veterinarian to come into his premises because he had just come from a farm where the disease had been confirmed. Another farmer did allow him to come in and inspect—the disease broke out in the farm 5 days later. However, this was the only occasion where a visit by a veterinarian who had been involved in infection was followed by the disease 5 or 6 days later.

SUMMARY

1. Foot-and-mouth disease broke out on three pig farms of the Spetchley area of Worcestershire after the virus had been carried to them in skimmed milk.

2. A study of the natural features of the area and of the climatic conditions during the outbreak show that the wind could have carried the virus from one or other of these three to twenty-six others of the total thirty-two farms infected.

3. Bringing the cattle from the fields and keeping them in their sheds offered no protection. Nine farmers did so but the only effect was that the onset of infection was delayed for a day or so.

4. The direction in which cow-sheds faced likewise made no difference. Openings in the walls for ventilation obviously allowed virus to be carried inside.

Mr J. S. Needham, M.R.C.V.S., first showed me that the wind could have been responsible for the spread of the disease in the Spetchley outbreak. I am glad to be able, after my study, to support his view. I am indebted to Mr V. Harris, Officer-in-charge, R.A.F. Meteorological Station, Pershore, Worcestershire, for the climatic details.

REFERENCE

Report of the Departmental Committee on Foot-and-mouth Disease 1952–54 (*The Gowers Report*). H.M.S.O. 1960.

APPENDIX 1

Wind directions and force Spetchley/Pershore area of Worcestershire from 14 *November to* 2 *December* 1967

14. xi. 67	Few light westerlies, otherwise 210°/260° light to moderate all day.
15. xi. 67	00.01–20.00 hr. 230°/250° light to moderate. At 21.00 hr. 270° moderate. 21.00–24.00 hr. 310°/340° moderate.
16. xi. 67	00.01–17.00 hr. 340°/360° light occasionally moderate. Very variable. 17.00–24.00 hr. calm.
17. xi. 67	00.01–11.00 hr. calm. 11.00–17.00 hr. 070°/100° light. 17.00–20.00 hr. 020°/060° light. 20.00–24.00 hr. calm.
18. xi. 67	00.01–12.00 hr. 010°/030° light. 12.00–21.00 hr. 020/070° light. 21.00–24.00 hr. 090°/120° light.
19. xi. 67	00.01–24.00 hr. 040°/100° light.
20. xi. 67	00.01–11.00 hr. 080°/110° light. 11.00–20.00 hr. 040°/070° light. 20.00–24.00 hr. 040°/360° light.

21. xi. 67	00.01–24.00 hr. 040°/070° light.
22. xi. 67	00.01–24.00 hr. 060°/100° light.
23. xi. 67	00.01–09.00 hr. 100°/130° light. 09.00–12.00 hr. 150°/180° light. 12.00–15.00 hr. calm. 12.00–24.00 hr. 120°/170° light.
24. xi. 67	00.01–12.00 hr. 130°/180° light. 12.00–18·00 hr. calm. 18·00–24·00 hr. 220°/240° light.
25. xi. 67	00.01–18.00 hr. 020°/070° light after early calm. 08.00 hr. 220°/250° light.
26. xi. 67	00.01–07.00 hr. 220°/250° light. 07.00–11.00 hr. calm. 11.00–13.00 hr. 260° light. 13.00–17.00 hr. 310° light 17.00–24.00 hr. 220°/250° light.
27. xi. 67	00.01–24.00 hr. 210°/230° light.
28. xi. 67	00.01–10.00 hr. 230° light to moderate. 10.00–24.00 hr. 270°/280° moderate.
29. xi. 67	00.01–11.00 hr. 260°/290° light to moderate. 11.00–17.00 hr. 280°/310° moderate. 17.00–20.00 hr. 270° light. 20.00–24.00 hr. 200°/220° light.
30. xi. 67	00.01–24.00 hr. 200°/230° light.
1. xii. 67	00.01–10.00 hr. 260° light. 10.00–19.00 hr. 360°/020° light. 19.00–24.00 hr. calm.
2. xii. 67	00.01–21.00 hr. calm. 21.00–24.00 hr. 260°/280° light.

Interpretation

Calm = calm smoke rises vertically. Light air = 1–3 knots (smoke drift can be seen). Light breeze = 4–6 knots (wind felt on face, wind vane is moved, leaves rustle). Gentle breeze = 7–10 knots (leaves and small twigs in constant motion, a light flag is extended). Moderate breeze = 11–16 knots (dust and loose paper raised).

APPENDIX 2

Rainfall in the Spetchley/Pershore area of Worcestershire from 14 November to 2 December 1967

Date	Rainfall (mm.)	Duration (hr.)	Date	Rainfall (mm.)	Duration (hr.)
14. xi. 67	1·9	2·1	24. xi. 67	—	—
15. xi. 67	2·1	1·8	25. xi. 67	0·5	1·0
16. xi. 67	Tr.	—	26. xi. 67	—	—
17. xi. 67	—	—	27. xi. 67	1·1	1·9
18. xi. 67	—	—	28. xi. 67	1·2	1·2
19. xi. 67	—	—	29. xi. 67	Tr.	—
20. xi. 67	Tr.	—	30. xi. 67	0·5	1·3
21. xi. 67	—	—	1. xii. 67	—	—
22. xi. 67	—	—	2. xii. 67	Tr.	—
23. xi. 67	—	—			

APPENDIX 3

Daily hours of sunlight in Spetchley/Pershore area of Worcestershire from 14 *November to* 2 *December,* 1967

Date	Sunlight (hr.)	Date	Sunlight (hr.)
14. xi. 67	3·7	24. xi. 67	0·8
15. xi. 67	0·8	25. xi. 67	0·8
16. xi. 67	2·9	26. xi. 67	1·0
17. xi. 67	Nil	27. xi. 67	Nil
18. xi. 67	0·6	28. xi. 67	Nil
19. xi. 67	0·2	29. xi. 67	Nil
20. xi. 67	4·9	30. xi. 67	Nil
21. xi. 67	Nil	1. xii. 67	Nil
22. xi. 67	7·1	2. xii. 67	Nil
23. xi. 67	3·1		

J. Hyg., Camb. (1969), **67**, 35
With 1 plate
Printed in Great Britain

Selection of *Staphylococcus aureus* in cultures from air samples

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

(*Received* 18 *May* 1968)

Although a good many selective culture media have been described for the isolation of *Staphylococcus aureus*, none of them has proved useful for the examination of cultures from air. This is partly because, in examining such cultures, one is trying to select the *S. aureus* from among a great variety of other members of the *Micrococcaceae*. Media depending on a high concentration of sodium chloride (e.g. Chapman, 1944), which are principally inhibitory for enterobacteria and similar Gram-negative bacilli, are not, therefore, of much use. Other media, such as those containing potassium tellurite (Zebovitz, Evans & Niven, 1955) or polymyxin B (Davis & Davis, 1965), though often satisfactory for examination of material from infected lesions or carrier sites, prove too inhibitory for the isolation of staphylococci from air. It has been a common experience that bacteria are often difficult to cultivate when collected from the airborne state. The recent studies on the taxonomy of staphylococci and micrococci published by Baird-Parker (1963, 1965) suggest a new approach, for he showed that *S. aureus* is able to grow at a higher temperature than most of the micrococci. Moreover members of the genus *Staphylococcus* are able to grow under anaerobic conditions, while those of the genus *Micrococcus* cannot, and other studies have shown that a substantial proportion of the airborne cocci fall into *Micrococcus* (Corse & Williams, 1968). We therefore explored the use of anaerobic culture at 41° C. as a selective method for the isolation of *S. aureus*. After a number of preliminary experiments with pure cultures, which need not be discussed here, all our studies were made by the exposure of culture plates containing the media under test in the wards of St Mary's Hospital.

METHODS

Air sampling

Culture plates 14 cm. in diameter were exposed in hospital wards for periods varying between 8 and 16 hr. The plates were stacked in the holder illustrated in Plate 1, which provides about 5 cm. separation between plates. Preliminary experiments showed that there were no significant differences in the numbers of colonies developing on the four plates in the stack. The culture plates with the different media were placed at random on the four layers of the stack. One or more stacks of plates were exposed each day but each stack included at least one 'control' plate.

The culture media used are listed in Table 1.

The 14 cm. diameter culture plates were poured with about 80 ml. of medium to give thick plates which resist desiccation during exposure in the ward.

For anaerobic incubation the plates were placed in 20 l. stainless-steel milk churns (R. J. Fullwood and Bland Ltd.) with a metal tube through the lid for evacuating the air, and three cold-catalyst sachets (Baird and Tatlock Ltd.), two at the top and one midway down the churn.

Table 1. *Composition of culture media*

	Control TY	Baird-Parker (Oxoid CM 275) BP	Modified Baird-Parker MBP
Tryptone	10 g.*	10 g.	10 g.*
Lab-Lemco beef extract	—	5 g.	—
Yeast extract	5 g.†	1 g.	5 g.†
K_2HPO_4	5 g.	—	—
NaCl	5 g.	—	—
Sodium pyruvate	—	10 g.	10 g.
Lithium chloride	—	5 g.	5 g.
Glycine	—	12 g.	12 g.
Agar	15 g.†	20 g.	15 g.†
1 % phenolphthalein diphosphate	10 ml.	10 ml.	10 ml.
Horse serum	50 ml.	—	50 ml.
Distilled water to	1000 ml.	1000 ml.	1000 ml.

*Oxoid brand. †Difco brand.

Further media were constituted as follows: TYG was TY + 1 % glucose, BPG was BP + 1 % glucose, BPS was BP + 5 % horse serum, TYM was TY + 1 % mannitol.

NOTE. The Oxoid CM 275 medium is based on Baird-Parker's (1962) description.

Examination of cultures

After incubation the plates were exposed to ammonia vapour and the bright pink colonies counted. All, or a sample, of these were subcultured for coagulase testing; coagulase-positive strains are referred to as *Staphylococcus aureus*. The total number of *S. aureus* colonies on the plates was estimated from the proportion of the sample found to be coagulase-positive.

RESULTS

In each experiment we had the results from the parallel exposure of one or more test media and one or more control plates on each of a series of days. The total number of colonies from any series of test plates was compared with that from the series of an equal number of control plates exposed simultaneously. Totals were also derived for *S. aureus* colonies. Thus in Expt. 1 (Table 2) seventeen plates of TYM medium were exposed on a total of 5 days and incubated anaerobically at 37° C. for 48 hr.; an equal number of control TY plates were exposed simultaneously and incubated aerobically at 37° C. for 18–20 hr. On the latter plates we counted a total of 44,284 colonies of which 1802 were estimated to be *S. aureus*. The counts of total colonies and *S. aureus* on the TYM plates were 60 and 93 % respectively of the control counts.

Table 2. *Comparison of various selective media for air samples*

Expt. no.	Test conditions*: Medium (see Table 1)	Incubation: Temp. °C.	Incubation: Time hr.	No. days of test	No. plates	Total colonies: On TY control†	Total colonies: On test as % of control	*Staphylococcus aureus*: On TY control†	*Staphylococcus aureus*: On test as % of control
1	TYM	37	48	5	17	44,284	60	1,802	93‡
	TYM	41	48	5	17	45,530	31	1,854	77‡
2	TY	41	48	7	22	61,688	22	243	97‡
	TYG	41	48	7	22	61,688	25	243	96
3	TYG	41	48	10	28	83,301	31	447	81
	BP	41	48	10	28	83,301	17	447	54‡
	BPG	41	48	10	28	83,301	16	447	45‡
4	TYG	41	48	9	28	55,733	34	161	96
	BPS	41	48	9	28	55,733	34	161	104
	MBP	41	48	9	28	55,733	28	161	103
5	TYG	41	24	6	24	50,071	22	824	92
	BPS	41	24	6	24	50,071	19	824	78‡
	MBP	41	24	6	24	50,071	23	824	76‡
6	TYG 8 hr. exp.	41	24	18	44	101,028	26	2,872	90
	TYG 16 hr. exp.	41	24	18	44	139,410	34	5,752	103

* All test media were incubated anaerobically.

† Control TY plates were incubated aerobically at 37° C. for 18–20 hr.

‡ *S. aureus* colonies were small and the phosphatase reactions difficult to read on these media.

Experiment 1 showed that with the mannitol agar (TYM) plates there was a substantial advantage from anaerobic incubation at 41° C. for the suppression of the general air flora; however, the *Staphylococcus aureus* colonies, especially on the 41° C. plates, were very small. In Expt. 2 both the media tested gave a good numerical yield of *S. aureus* but the colonies on the control (TY) medium incubated at 41° C. were very much smaller than those on the glucose-containing (TYG) medium incubated at 41° C.

Various experiments were carried out with Baird-Parker's medium, alone, enriched with glucose or serum, or modified as shown in Table 1 (Expts. 3–5). Although in some cases the results appear from the counts to have been satisfactory, the phosphatase reaction was often difficult to read, and there were unexplained variations in the proportion of the *S. aureus* colonies that grew on the medium, as is seen in the comparison of the three experiments cited in Table 2.

The TYG medium incubated at 41° C. anaerobically for 48 hr. seemed to yield, reasonably consistently, *S. aureus* counts at about 90 % of the number on the control plates (TY incubated aerobically), while reducing the total bacterial count to around 20–30 % of the control count. Further experiments (Expts. 5, 6) indicated that 24 hr. incubation was equally good.

In Expts. 1–5 all the plates were exposed in the wards for 12 hr. A final test was made in which the plates exposed for 8 hr. were compared with those exposed for 16 hr., the latter being a convenient 'overnight' exposure time. As will be seen, the yield of *S. aureus* on the 16 hr. TYG plates incubated anaerobically at 41° C. for 24 hr. was virtually the same as that on the control TY plates (incubated at 37° C. aerobically for 24 hr.); on the 8 hr. plates the *S. aureus* count was slightly lower than on the corresponding control plates. The total colony count was less reduced on the 16 hr. plates than on the 8 hr. plates.

COMMENT

The enumeration of *Staphylococcus aureus* in the air of hospital wards may be of some value for routine monitoring in hospital hygiene (Alder & Gillespie, 1964) and also in the exploration of the hygienic consequences of particular sorts of hospital design or practice (Lidwell *et al.* 1966; Williams, 1967). A truly selective medium for collection of *S. aureus* from the air would be a great advantage for either of these uses, but none of the selective media devised in the past has been found suitable. The method described here, involving the use of a relatively simple medium enriched with 1 % glucose and 5 % horse serum incubated anaerobically at 41° C., appears to us to be a distinct advance over the previous methods. Phosphatase-positive colonies are not by any means always easy to distinguish, and a method that substantially reduces the number of colonies that have to be scanned certainly eases the reading of the plates. The technique described has now been in regular use in a survey in the isolation ward of St Mary's Hospital for a period of several months and has greatly reduced the labour and time involved in searching the culture plates for *S. aureus*.

LYNN HARDING AND R. E. O. WILLIAMS

(*Facing p. 39*)

SUMMARY

A tryptone–yeast extract medium enriched with glucose and serum incubated anaerobically at 41° C. was found to give a good yield of *Staphylococcus aureus* from air samples while suppressing the growth of 70–80 % of the other airborne bacteria.

Our thanks are due to Miss S. M. Taber and Miss Patricia Wall, the Sisters in the wards where the air samples were collected, for their help. The investigation was financed from the Ministry of Health grant to St Mary's Hospital for the support of clinical research.

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.

BAIRD-PARKER, A. C. (1962). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J. appl. Bact.* **25**, 12.

BAIRD-PARKER, A. C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.* **30**, 409.

BAIRD-PARKER, A. C. (1965). The classification of staphylococci and micrococci from world-wide sources. *J. gen. Microbiol.* **38**, 363.

CHAPMAN, G. H. (1944). The isolation of pathogenic staphylococci from feces. *J. Bact.* **47**, 211.

CORSE, J. & WILLIAMS, R. E. O. (1968). Antibiotic resistance of coagulase-negative staphylococci and micrococci. *J. clin. Path.* **21**, 722.

DAVIS, N. A. & DAVIS, G. H. G. (1965). Selective culture of coagulase-positive staphylococci. *J. Path. Bact.* **89**, 380.

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**, 321.

WILLIAMS, R. E. O. (1967). Airborne staphylococci in the surgical ward. *J. Hyg., Camb.* **65**, 207.

ZEBOVITZ, E., EVANS, J. B. & NIVEN, C. F. JR. (1955). Tellurite-glycine agar: a selective plating medium for the quantitative detection of coagulase-positive staphylococci. *J. Bact.* **70**, 686.

EXPLANATION OF PLATE

Plate holder used for the simultaneous exposure of four culture plates. One of the vertical strips moves laterally to facilitate insertion of the plates and removal of the lids.

J. Hyg., Camb. (1969), 67, 41
Printed in Great Britain

Chick embryo lethal orphan (CELO) virus as a possible contaminant of egg-grown virus vaccines

By J. S. OXFORD and C. W. POTTER
Virus Research Laboratory, Lodge Moor Hospital, Sheffield and Department of Medical Microbiology, University of Sheffield

(*Received* 31 *May* 1968)

INTRODUCTION

Viruses which are able to establish persistent subclinical infections in their natural hosts may present serious problems in the preparation of vaccines. These viruses may be present, undetected by conventional virus isolation techniques, in tissue cultures prepared from laboratory animals. Of particular importance in this respect are viruses with oncogenic potential and it is now well established that formaldehyde-inactivated poliovirus vaccines made in monkey renal-cell cultures may contain an oncogenic virus of simian origin, SV40 virus (Goffe, Hale & Gardner, 1961; Gerber, 1967). In addition, viruses of the avian leucosis group are common contaminants of fertile hens' eggs and such viruses have been detected in live attenuated yellow fever vaccines prepared in eggs (Harris *et al.* 1966).

Since fertile eggs are employed in the production of a number of different attenuated or killed virus vaccines, latent viruses of chickens and their eggs merit detailed investigation. Such a virus is Chick Embryo Lethal Orphan (CELO) virus, which was first isolated by serial passage of allantoic fluid from apparently normal fertile eggs (Yates & Fry, 1957). Sero-epidemiological studies have shown that CELO infection is widespread in flocks of fowls in the U.S.A. and Japan (Yates & Fry, 1957; Kawamura *et al.* 1963). More recently CELO virus has been isolated in England from eggs laid by an apparently normal flock with exceptionally high egg production (Cook, 1968). Sarma, Huebner & Lane (1965) have described the induction of fibrosarcomas in hamsters inoculated with CELO virus when newborn and the oncogenic properties of the virus have been confirmed in this laboratory with two different strains of CELO virus (Schild, Oxford & Potter, to be published). CELO virus is a potential contaminant of egg-prepared vaccines and because of its stability (Petek, Felluga & Zoletto, 1963) may survive vaccine virus inactivation procedures.

In the present study the inactivation kinetics of CELO virus in the presence of 1/4000 formaldehyde have been investigated. This concentration of formaldehyde has been used in the preparation of certain killed virus vaccines (Gerber, Hottle & Grubbs, 1961). Further, we have tested a number of sera collected from flocks of chickens for the presence of CELO virus neutralizing antibody in order to investi-

gate whether infection with the virus is widespread in England. Finally, we have searched for CELO virus antibody in the sera of persons who have received killed influenza vaccines.

METHODS

Virus

The 'Phelps' strain of CELO virus was supplied by Dr J. S. Garside, Houghton Poultry Research Station, Huntingdon. The virus was diluted 1/100 in phosphate buffered saline and 0·2 ml. inoculated allantoically in 7-day-old fertile hens' eggs. Allantoic fluids were harvested after 5 days incubation at 35° C. and stored at − 70° C. in ampoules. The virus pool had a titre of $10^{9\cdot0}$ ELD50/ml. in eggs and $10^{8\cdot5}$ TCID50/ml. in tissue cultures of chick kidney.

Serum samples

Chicken sera were kindly supplied by Dr J. E. Wilson, Ministry of Agriculture, Fisheries and Food, Veterinary Laboratory, Lasswade, and Dr D. P. McHugh, Pfizer Co., Sandwich, Kent. Other sera were from our own flock of hens.

Rabbit sera were from animals immunized with three weekly intravenous injections of the stock CELO virus egg allantoic fluid pool. The rabbits were bled 2 weeks after the final injection.

The human sera were collected during trials of various influenza vaccines.

R.A.F. Halton Trial. Sera were taken 18 months after the subcutaneous injection of 0·25 ml. of a bivalent influenza vaccine containing 2000 haemagglutinating units of influenza A2/Singapore/1/57 and B/England/939/59 viruses. The vaccines contained either vegetable-oil adjuvant (A 65) or Drakeol no. 6 mineral-oil adjuvant.

Reed Paper Group trial, Maidstone. The sera were collected 12 months after subcutaneous injection of bivalent vaccines containing influenza A2/Jap/170/62 and B/Maryland/1/59 viruses. Volunteers were given either an aqueous (saline) vaccine containing 500 haemagglutinating units of each virus, or an adjuvant vaccine (adjuvant A 65, lot 140) containing 250 haemagglutinating units per 0·5 ml. dose.

Sera were also tested which had been collected in 1961–3 during trials of attenuated poliomyelitis vaccines. For the purpose of the present study these were designated 'normal' sera, there being no known history of administration of virus egg vaccines to these individuals.

Tissue cultures

Chick kidneys were removed aseptically from fowls aged 2–4 weeks, trypsinized and seeded into tubes at a concentration of 0·1 ml. centrifuged cell pack per 100 ml. of medium. Chick embryo fibroblast cells were prepared by mincing and trypsinizing decapitated embryos from 10-day-old fertile hens' eggs and seeded into tubes at a concentration of 0·1 ml. of centrifuged cell pack per 100 ml. of medium. The cell growth medium was Eagle's minimal essential medium (M.E.M.) containing 10 % inactivated calf serum and 0·44 g./l. sodium bicarbonate. The maintenance medium contained 5 % inactivated calf serum and 0·88 g./l. sodium bicarbonate in M.E.M.

Neutralization tests

Equal volumes of serum dilution and CELO virus containing 100 TCID 50 were incubated for 1 hr. at room temperature and 0·2 ml. inoculated in each of four chick kidney tube tissue cultures. Neutralization end-points were taken as the highest serum dilution completely inhibiting virus cytopathic effect after 7 days incubation at 35–36° C.

Formaldehyde inactivation of CELO virus

Formaldehyde was diluted to 1/4000 in phosphate buffered saline in stoppered conical flasks and CELO virus added to give a final concentration of $10^{7\cdot5}$ TCID 50/ml. The flasks were shaken periodically to wash any virus from the vessel walls. Samples were withdrawn at various time intervals, the formaldehyde neutralized with sodium bisulphite and the fluids titrated for CELO virus in chick kidney tissue cultures. A control flask containing virus but no formaldehyde was tested in parallel and the experiment carried out at 4° C. and 36° C.

Table 1. *Comparison of chick kidney cells and chick embryo fibroblast cells for quantitation of CELO virus*

(Titre of virus ($\log_{10}$ TCID 50/ml.) and C.P.E. after infection of cells with different virus multiplicities.)

Time after infection of cultures (days)	1·0 TCID 50 per cell*		0·01 TCID 50 per cell	
	Chick kidney	Chick embryo fibroblast	Chick kidney	Chick embryo fibroblast
0	4·5†(0)	4·5 (0)	1·5 (0)	1·5 (0)
½	6·5 (±)	5·3 (0)	1·8 (0)	1·5 (0)
1	7·8 (+ + +)	5·5 (0)	3·5 (0)	1·8 (0)
2	7·8 (+ + + +)	5·5 (±)	5·7 (−)	2·8 (0)
4	8·5 (+ + + +)	5·8 (+ + +)	7·5 (− + + +)	4·5 (0)
7	8·5 (+ + + +)	5·8 (+ + + +)	7·5 (− + + +)	5·3 (+)
10	8·5 (+ + + +)	5·8 (+ + + +)	7·5 (+ + + +)	5·3 (+ + +)

+ + + +, + + +, + +, +, Cytopathic effect (C.P.E.) in 100 %, 75 %, 50 % and 25 % of cells respectively.

* virus adsorbed for 2 hr. at room temperature, cells washed 3 times to remove unadsorbed virus and incubated at 36° C.

† Titre of virus expressed as $\log_{10}$ TCID 50/ml. from three pooled tissue culture tubes.

RESULTS

In preliminary experiments we compared the relative sensitivity of chick kidney cells and chick embryo fibroblast cells for the detection of CELO virus cytopathic effects and for growth of the virus. At two multiplicities of virus infection (0·01 and 1·0 TCID 50 virus per cell) cytopathic changes were noted earlier, and virus titres in the supernatant fluids reached higher levels, in chick kidney cell cultures as compared to chick embryo fibroblasts (Table 1). In addition, a single pool of CELO virus was titrated in parallel in tenfold dilution steps using these two types of cell. The titre of the virus read at 10 days by cytopathic end-point was $10^{8\cdot3}$ TCID 50/ml. in chick kidney cells and $10^{6\cdot5}$ TCID 50/ml. in chick embryo fibroblast cells. Chick kidney cells were therefore used for all subsequent studies with CELO virus.

Formaldehyde inactivation of CELO virus

The inactivation of CELO virus in the presence and absence of 1/4000 formaldehyde is shown in Fig. 1. In control fluids, containing no formaldehyde, the infectivity titre of CELO virus did not decline significantly after 14 days incubation at 4° or at 36° C. In contrast, virus inactivation at 36° C. in the presence of 1/4000 formaldehyde was relatively rapid, and no CELO virus was detected after 24 hr. incubation. Inactivation of CELO virus by formaldehyde at 4° C. was less efficient; $10^{2\cdot5}$TCID 50/ml. of virus was still present in the fluids after 14 days incubation.

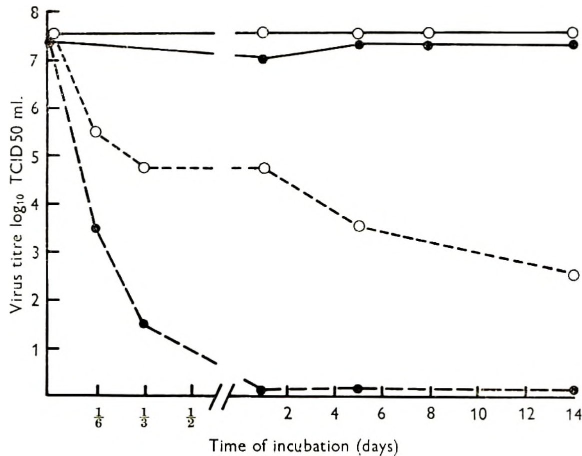


Fig. 1. Formaldehyde inactivation of CELO virus. ○——○, PBS at 4° C., ●——●, PBS at 35–36° C., ○– – –○, formaldehyde 1/4000 at 4° C., ●– – –●, formaldehyde 1/4000 at 35–36° C.

Neutralizing antibodies for CELO virus in sera

Chicken sera from a number of flocks were tested at a dilution of 1/8 for the presence of neutralizing antibody to CELO virus and the results are shown in Table 2. Neutralizing antibody was detected in a proportion of each group of chicken sera tested ranging from 20 % to 88 %. In a further experiment twenty-nine chicken sera from two groups were titrated in twofold dilution steps to find the range of neutralizing antibody titres (Table 3). CELO virus neutralizing antibody titres ranged from 1/10 to 1/640 and the modal titre of neutralizing antibody was between 1/40 and 1/160.

No CELO virus neutralizing antibody was detected in the 142 normal human sera examined at a dilution of 1/8 from a random section of the population. Similarly, no CELO virus neutralizing antibody was detected in the sera tested from 229 individuals who had been immunized with inactivated influenza virus vaccines prepared by two different manufacturers. Rabbits which were immunized with three successive doses of CELO virus developed high titres of neutralizing antibody ranging from 1/512 to 1/2048. This suggested that CELO virus was a potent antibody inducer in species additional to its natural host.

Table 2. *CELO virus neutralizing antibody in human and animal sera*

Source of sera	No. tested	No. of sera with neutralizing antibody at serum dilution 1/8
A. Human sera		
After influenza vaccine, Maidstone trial	141	0
After influenza vaccine, R.A.F. Halton trial	88	0
Normal individuals: 10 months to 5 years of age	30	0
Normal individuals: 18 years of age and over	112	0
B. Chicken sera		
Pfizer, Kent	10	2
Lodge Moor, Yorks.	9	8
D945 Lasswade, Scotland	25	15
D801 Lasswade, Scotland	12	6
D.3, Lasswade Scotland	20	15
C. Rabbit sera		
Immunized with CELO virus	4	4

Table 3. *CELO virus neutralizing antibody in chicken sera*

No. of sera tested*	No. of sera with neutralizing antibody at indicated serum dilution							
	<1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
29	6	3	1	6	4	6	2	1

* Chicken sera from Lodge Moor and Lasswade D.3.

DISCUSSION

Previous studies with CELO virus have described the cytopathic effects of the virus growing on chick kidney cells (Chomiak, Luginbuhl & Helmboldt, 1961). However, chick embryo fibroblast cells are a more readily obtainable source of chick cells and were, therefore, compared with chick kidney cells for their relative sensitivity for the growth of CELO virus. The latter were found to be superior both for the growth and titration of CELO virus; higher titres of virus were produced and cytopathic changes occurred more quickly in chick kidney cells. Chick kidney cells therefore appear to be more sensitive for the detection of small quantities of CELO virus in vaccines, for example, than would be chick embryo fibroblast cells.

The inactivation kinetics experiments with allantoic fluids containing high initial infectivity titres of CELO virus indicated that no detectable virus survived the inactivation period of 24 hr. with 1/4000 formaldehyde at 36° C. However, the inactivation process at 4° C. was less complete and infective CELO virus was detected even after 14 days. The present formaldehyde inactivation studies with

CELO virus did not show a linear reaction. Thus, the major portion of the CELO virus population was inactivated in the first 8 hr. at 4° C. in the presence of formaldehyde. After this initial inactivation the titre of infective virus continued to decrease more slowly over the subsequent test period of 14 days. CELO virus appears to be more efficiently inactivated than another stable potential contaminant of certain vaccines, SV 40 virus; Gerber *et al.* (1961) demonstrated that some SV 40 virus infectivity was retained even after 14 days inactivation at 33° C. with 1/4000 formaldehyde.

The present survey of human sera for CELO virus neutralizing antibody failed to detect any serological evidence that persons who had received egg-prepared inactivated influenza virus vaccines were also injected concurrently with CELO virus. These negative results, however, do not demonstrate unequivocally that the original vaccines were free of CELO virus. Harris and his colleagues (1966) were able to demonstrate the contamination of yellow fever vaccine with an avian leukosis virus but were unable to detect any antibody to the latter virus in immunized volunteers. In contrast, antibody for an avian leukosis virus was detected after repeated immunization of chickens with these vaccines.

CELO virus-neutralizing antibody was detected in the serum of a proportion of hens in each of the five flocks tested. This suggests dissemination of the virus in England and Scotland. In addition, studies in another laboratory have also detected CELO virus neutralizing antibody in chicken sera in Great Britain. (D. A. McMartin, Veterinary Laboratory, Lasswade, Midlothian—personal communication.) Recently CELO virus has been isolated from eggs laid by an apparently normal flock in England (Cook, 1968). Similar studies in the U.S.A. and Japan have also noted a proportion of hens from different flocks with CELO virus neutralizing antibody (Yates & Fry, 1957; Kawamura *et al.* 1963). More studies are thus indicated to attempt CELO virus isolation from eggs and chickens, particularly in flocks used for vaccine production. It may then be possible to circumvent the problem of potential contamination of vaccines with this virus by only using eggs from virus free, seronegative flocks.

SUMMARY

The inactivation kinetics of CELO virus were studied in the presence of 1/4000 formaldehyde. Inactivation of the virus by formaldehyde at 4° C. was not complete after 14 days incubation. Formaldehyde inactivation at 36° C., however, was rapid and no virus was detected after 24 hr. incubation.

Neutralizing antibody to CELO virus was detected in 20–88 % of sera tested from five flocks of hens. This suggested dissemination of the virus in England and Scotland. However, no CELO virus neutralizing antibody at a serum dilution of 1/8 was detected in 142 normal human sera or in 229 sera from persons who had been immunized with egg grown, inactivated influenza virus vaccine.

We would like to thank Professor C. H. Stuart-Harris and Drs J. E. Wilson, D. A. Martin and D. Breeze for their help and their criticisms of the manuscript.

Dr G. C. Schild kindly supplied a number of the human sera used in the study. The study was financed in part by the National Fund for Research in Poliomyelitis and other Crippling Diseases and by the British Empire Cancer Campaign.

REFERENCES

CHOMIAK, T. W., LUGINBUHL, R. E. & HELMBOLDT, C. F. (1961). Tissue culture propagation and pathology of CELO virus. *Avian Dis.* **5**, 313.

COOK, J. K. A. (1968). Isolation of a CELO virus from fertile chicken eggs. *Vet. Rec.* **82**, 294.

GERBER, P. (1967). Patterns of antibodies to SV 40 in children following the last booster with inactivated poliomyelitis vaccines. *Proc. Soc. exp. Biol. Med.* **125**, 1284.

GERBER, P., HOTTLE, G. A. & GRUBBS, R. E. (1961). Inactivation of vacuolating virus (SV 40) by formaldehyde. *Proc. Soc. exp. Biol. Med.* **108**, 205.

GOFFE, A. P., HALE, J. & GARDNER, P. S. (1961). Poliomyelitis vaccines. *Lancet* i, 612.

HARRIS, R. J. C., DOUGHERTY, R. M., BIGGS, P. M., PAYNE, L. N., GOFFE, A. P., CHURCHILL, A. E. & MORTIMER, R. (1966). Contaminant viruses in two live virus vaccines produced in chick cells. *J. Hyg., Camb.* **64**, 1.

KAWAMURA, H., SATO, T., TSUBAHARA, B. & ISOGAI, S. (1963). Isolation of CELO virus from chicken trachea. *Natn. Inst. Anim. Hlth Q. Tokyo* **3**, 1.

PETEK, M., FELLUGA, B. & ZOLETTO, R. (1963). Biological properties of CELO virus: Stability to various agents, and electron microscopy study. *Avian Dis.* **7**, 38.

SARMA, P. S., HUEBNER, R. J. & LANE, W. T. (1965). Induction of tumours in hamsters with an avian adenovirus (CELO). *Science, N.Y.* **149**, 1108.

YATES, V. J. & FRY, D. E. (1957). Observations on a chicken embryo lethal orphan (CELO) virus. *Amer. J. vet. Res.* **18**, 657.

J. Hyg., Camb. (1969), **67**, 49
Printed in Great Britain

Importance of the carrier state as a source of *Staphylococcus aureus* in wound sepsis*

BY FRANK M. CALIA,†, EMANUEL WOLINSKY, EDWARD A. MORTIMER, JR, JEROME S. ABRAMS, AND CHARLES H. RAMMELKAMP, JR

From the Departments of Medicine, Pediatrics and Surgery, Cleveland Metropolitan General Hospital, and Case Western Reserve University School of Medicine, Cleveland, Ohio 44109.

(*Received* 11 *June* 1968)

Wound sepsis continues to be a significant problem in surgery despite the availability of potent antimicrobial agents. Average rates of wound sepsis are reported to be 5–10% (Williams *et al.* 1959; Jeffrey & Sklaroff, 1958), but vary from 1% to over 50% (Rogers, Duffy & Mou, 1965; Ketcham *et al.* 1962) depending on the population under consideration and the type of surgery performed. Factors known to be important include host resistance (Fekety, 1964), site and duration of operation and need for blood transfusion (Cohen, Fekety & Cluff, 1964), presence of infection elsewhere (Altemeier, Hummel & Hill, 1966), and contact with a carrier or infected person (Browne *et al.* 1959; Burke & Corrigan, 1961).

Regardless of these factors, wound infection or sepsis with *Staphylococcus aureus* cannot occur unless organisms are deposited in the wound. This might take place from several sources and by various mechanisms: blood borne from a remote lesion or the nasopharynx (Walter, 1958); direct implantation from contaminated hands, large droplets, fomites (Colbeck, 1960) or airborne droplet nuclei (Browne *et al.* 1959); or from contaminated skin at the incision site (Cole & Bernard, 1964). Initial contamination might occur in the operating room before wound closure or after the patient has been returned to the ward. The present study was designed to examine one facet of wound sepsis: self-contamination by patients who exhibit nasal and skin carriage of staphylococci preoperatively.

PLAN OF STUDY

Patients admitted to one male and one female general surgical ward were assigned to the study if there was a reasonable assurance of early operation plus easy accessibility of the wound for post-operative bacteriological studies and inspection. Thus, those for whom operation in the near future was not planned and certain orthopaedic and neurosurgical patients were not included in the study

* This investigation was conducted under the sponsorship of the Commission on Streptococcal and Staphylococcal Diseases, Armed Forces Epidemiological Board, and supported by the Office of the Surgeon General, Department of the Army, Washington, D.C. Reprint requests to Cleveland Metropolitan General Hospital, Cleveland, Ohio 44109 (Dr Wolinsky).

† Former Trainee in Infectious Diseases, supported by Grant 5 TO 1 AI 00180, National Institute of Allergy and Infectious Disease, Public Health Service.

group. Swabs from the anterior nares, axilla, umbilicus, and groin of the study patients were obtained on admission or soon thereafter and again at frequent intervals—usually every other day. After operation cultures were taken from the wound at the first dressing change and frequently thereafter. Cultures from the anterior nares were obtained weekly from the other patients on the ward. Dry cotton swabs were used for the nares and wounds, and swabs moistened in broth served to culture the other skin sites. The swabs were streaked immediately on mannitol salt agar and the plates were incubated at 37° C. for 48 hr. The amount of growth of *Staph. aureus* was roughly estimated by colony count and by graded evaluations from 1+ to 4+. Two to eight representative colonies were picked from each culture and identified by phage typing, antibiogram and, if necessary, coagulase testing. When the identity of two or more similar strains was in doubt, several more colonies from each culture were retested simultaneously. The phages employed were the conventional set of 22, with the addition of UC 18 and two others developed in this laboratory. Phage was used at 100 times the routine test dilution. With this technique, less than 10% of strains could not be typed.

Skin strips from the incision site secured with a double-blade knife at the beginning of the operation, and wound swabs taken immediately before skin closure, were obtained from many patients. The swabs were inoculated on blood agar, and into trypticase soy broth which was subcultured to a blood agar plate after 24 hr. of incubation. One-half of each skin strip was homogenized by means of a mortar and pestle and the remainder was minced into small pieces with sterile scissors. The minced pieces and the homogenate were cultured separately in trypticase soy broth which was then subcultured onto a blood agar plate after incubation for 24 hr.

A carrier was defined as a patient whose preoperative culture yielded more than one colony of a given phage type, or any number of a single strain on more than one occasion. Roughly half of the subjects had only a single set of cultures before operation.

Wound colonization was defined as the recovery of *Staph. aureus* by culture from the postoperative wound swabs, except that single colonies of a given strain isolated only once were not considered to be significant. Wound sepsis was defined as the presence of purulent drainage from which *Staph. aureus* could be cultured. Minor collections of pus confined to a stitch orifice were not considered to be wound sepsis.

Statistical significance was determined by the χ^2 test, and a P value of less than 0·05 was considered to be significant.

RESULTS

Two hundred and sixty-nine patients were studied over a 2-year period. Ninety-six, or 36%, were carriers of *Staph. aureus* before operation; 82 patients had positive cultures on admission and 14 acquired the organism in the hospital before operation. The relationship of the carrier state to wound colonization and wound sepsis is shown in Table 1. *Staph. aureus* was isolated from the wounds of 63 patients (23%) during the postoperative period. Colonization occurred more than twice as

often in carriers (37 %) as in non-carriers (16 %), and the highest rate (56 %) was found among skin carriers. The difference between 37 % and 16 % is significant, with a P value of < 0·001.

Table 1. *Relationship of the carrier state to wound colonization and sepsis*

Carriage pre-op.	No. of patients	Wounds colonized No.	Wounds colonized %	Wounds septic No.	Wounds septic %
Non-carriers	173	27	16	16	9
Carriers					
Nose only	51	11	22	6	12
Skin	45*	25†	56	10‡	22
Total	96	36	37	16	17
All patients	269	63	23	32	12

* Ten were carriers on skin only. † Three were carriers on skin only.
‡ One was a carrier on skin only.

Table 2. *Relationship of carrier strain to wound colonization and sepsis*

	Patients with wound colonization	Patients with wound sepsis
Total	63	32
Non-carriers	27	16
Carriers	36	16
With carrier strain only	27	13
With carrier plus exogenous strain	6	3
With exogenous strain only	3	0

The total rate of wound sepsis was 12 %. It was almost twice as great in carriers (17 %) as in non-carriers (9 %), but this difference is not statistically significant. There was a significant difference, however (P value of 0·04), in the wound sepsis rate in skin carriers (22 %) as compared to the non-carriers. Nasal carriage alone, without skin carriage, did not result in a significant increase in wound colonization or wound sepsis in comparison to the non-carrier group. Likewise, carriage on the skin only was not demonstrated to influence rates of wound colonization and sepsis, though the numbers involved were small. Colonization occurred in 3 of the 10 skin-only carriers, and wound sepsis occurred in 1 of 10. This finding suggests that combined nasal and skin carriage might be the important factor, and this in turn might be a reflexion of density of organisms at carrier sites.

The relationship between the strains carried in the nose or on the skin and those which colonized wounds is presented in Table 2. Of the 36 pre-operative carriers whose wounds were colonized, 27 exhibited only the carrier strain and six others yielded both the carrier strain and an exogenous strain from the wound. Therefore,

it is apparent that when the wounds of preoperative carriers became colonized with *Staph. aureus* it was usually the carrier strain that was recovered. This relationship is even more striking in the case of wound sepsis. Among 16 septic wounds in the carrier group, the carrier strain was recovered in all instances, although in three cases an exogenous strain was also cultured from the wound.

In the total study group, carriers and non-carriers, there were 63 patients whose wounds were colonized, of which 33 yielded the carrier strain; and in 32 whose wounds were septic 16 yielded the carrier organism. Thus, the carrier strain accounted for approximately half of the instances of colonization and sepsis.

The data were analysed to ascertain whether or not there was a difference in wound colonization and sepsis rates between non-carriers versus carriers infected with exogenous strains. Whereas these rates were 16 % for colonization and 9 % for sepsis in non-carriers, the corresponding rates for infection with exogenous strains among carriers were 9 % (9 of 96) and 3 % (3 of 96). These differences in rates for colonization and for sepsis were not statistically significant. It should be remembered, however, that in the latter group 6 of the 9 colonized wounds and all 3 of the septic wounds contained the carrier strain as well as an exogenous strain of *Staph. aureus*. Thus, there was no evidence for either interference as suggested by Shinefield *et al.* (1963) or a predisposition to exogenous staphylococcal infections in carriers.

Table 3. *Wound colonization and sepsis related to carriage on normal* v. *broken skin**

	Number of skin carriers	No. of wounds colonized	No. of wounds septic
Total	45	25 (56 %)	10 (22 %)
On normal skin	28	14 (50 %)	5 (18 %)
On broken skin	17	11 (65 %)	5 (29 %)

* For example, vascular leg ulcers, burns, dermatitis, etc.

The association between wound sepsis and infection at a remote site is well known (Altemeier *et al.* 1966). An attempt was made to ascertain whether wound colonization and sepsis occurred more commonly among patients who carried *Staph. aureus* on broken skin as opposed to carriage on normal or intact skin. Examples of broken skin included chronic dermatitis, ulceration of the legs associated with vascular insufficiency, draining sinuses and burns. It should be pointed out that the surgical incisions were not made through these areas. The results presented in Table 3 suggest that carriage on broken skin did not increase wound colonization or sepsis rates compared to carriage on normal skin, but the number of cases was too small for satisfactory statistical analysis.

Using quantitative techniques, White (1961) has documented a greater incidence of skin carriage of *Staph. aureus* among individuals with heavy nasal carriage than among those with light carriage; he also showed a direct relationship between the number of organisms in the nose and dissemination into the air. The data from the present study were analysed in an attempt to relate quantity of nasal carriage to skin contamination, wound colonization and wound sepsis. The results are shown

in Table 4. Although interpretation of these data is difficult because there were ten patients who had infected skin lesions and were not nasal carriers, it was not possible to relate quantity of nasal carriage to positive skin cultures. The only significant difference was in the rate of wound sepsis in that the profuse nasal carriers showed a rate of 31 % as compared with 8 % in those with moderate, light or no nasal carriage ($P = 0{\cdot}01$).

Table 4. *Quantity of preoperative nasal carriage related to skin carriage, wound colonization and sepsis*

Nasal carriage	No. of patients	Skin carriers		Wounds colonized*		Wounds septic*	
		No.	%	No.	%	No.	%
Heavy	36	17 (5)†	47	16	44	11	31
Moderate	31	8 (2)	26	9	29	3	10
Light or none	29	20 (10)	69	8	30	2	7

* With carrier strain.

† Numbers in parentheses represent patients with carriage on broken skin, some of whom carried on intact skin as well.

Table 5. *Results of cultures of operating room specimens obtained from* 74 *patients*

	No.	Sterile	*Staph. aureus*
Wound swabs	48	13	0
Skin strips	60	14	9*

* Four patients were pre-operative carriers; none had same strain in skin strip. Eight patients had subsequent cultures, and none were colonized later with skin-strip strain.

The results of cultures from the specimens obtained in the operating room are given in Table 5. Only 13 of the 48 wound swabs were sterile but in no instance was *Staph. aureus* recovered. The cultures usually revealed common skin contaminants such as *Staph. albus*, diphtheroids, and α-haemolytic or non-haemolytic streptococci. The majority of the 60 skin-strip specimens yielded similar skin contaminants, but in nine instances *Staph. aureus* was isolated. Four of these patients were preoperative carriers but none carried in the nose or on the skin the same strain that was found in the skin strip. Eight of the nine were cultured frequently enough postoperatively for adequate follow-up, but subsequent wound colonization with the strain found in the skin strip was not observed. Subsequent observations, involving an additional 97 patients, have revealed a single instance of a carrier strain isolated from an incisional wound swab and later causing wound sepsis.

DISCUSSION

This study was designed to examine the importance of the patient himself as a source of the staphylococci that infect wounds. A divergence of opinion exists in the literature concerning the relationship between carrier strains of *Staph. aureus*

and those recovered from the wound. A significant correlation has been found by some investigators (Williams *et al.* 1959; Colbeck, 1960; Burke, 1963), but others (Rogers *et al.* 1965; Browne *et al.* 1959; Bullock *et al.* 1964) have not. Williams *et al.* (1959), Weinstein (1959), McNeill, Porter & Green (1961), Williams *et al.* (1962), Ketcham *et al.* (1962), Lindbom, Laurell & Grenvik (1967) and Lindbom & Laurell (1967*a*, *b*) reported a higher incidence of wound sepsis among carriers, but Rogers *et al.* (1965), Bullock *et al.* (1964), Browne *et al.* (1959), Lowden, Vaithilingham & Milne (1962), Bassett *et al.* (1963) and Moore & Gardner (1963) did not find this to be true.

In the present study definite association was found between staphylococcal carriage and subsequent wound colonization. Furthermore, the presence of combined nasal and skin carriage was associated with a higher colonization rate than nasal carriage alone. Although the difference in sepsis rates between non-carriers and the total group of carriers was not statistically significant, there was a significant difference in rates between non-carriers and those who carried the organism on the skin. Since the majority of skin carriers were nasal carriers as well, the important factor was apparently the combination of skin and nasal carriage.

The significance of the carrier state is also emphasized by the finding that the homologous strain appeared in the wounds of 33 of the 36 carriers whose wounds were colonized. Even among the nine carriers whose wounds were colonized with an exogenous strain, in six the carrier strain was also present. More significantly, septic wounds in the 16 carriers yielded the carrier strain in each instance. The carrier strain accounted for roughly half of all instances of wound colonization and sepsis in the entire study group. One can not rule out the possibility that some of the wounds were infected with strains originating from other sources in the ward that happened to be similar to the patient's carrier strain. In most cases, however, the carrier strains were sufficiently distinct from the monitored ward strains to make this possibility very remote.

Infection at a site remote from a surgical incision has been implicated as a causal factor in wound sepsis (Altemeier *et al.* 1966). In the present study, when the skin carriers were separated into those whose organisms were isolated from intact skin and from broken skin, no statistically significant difference in wound colonization or sepsis could be found, but the number of cases involved was too small to justify a definite conclusion.

It has been shown by White (1961) and Ehrenkranz (1964) that skin colonization and airborne dissemination of *Staph. aureus* is a function of the number of organisms present in the nose. The higher incidence of wound colonization and sepsis in skin carriers in this study might be related primarily to heavy nasal carriage. It may be hypothesized that the heavy nasal carriers would not only tend to become skin carriers but would also be most likely to develop colonization and sepsis of the wound. It is not possible from the data accumulated so far to establish a relationship between wound colonization and quantity of nasal carriage. Wound sepsis, however, was found to occur more frequently in profuse nasal carriers.

Browne *et al.* (1959) and Roberts (1965) suggested that isolation of pathogenic organisms from surgical incisions might presage subsequent wound sepsis with that

organism. Howe & Marston (1962), however, were unable to associate wound sepsis with *Staph. aureus* isolated from the incision at the time of closure. Wise, Sweeney, Haupt & Waddell (1959) found that skin strips removed from the incision site some time during surgery were usually not sterile and that 11 % contained *Staph. aureus*, but these organisms did not seem to initiate wound sepsis. The results of the present study were similar to those of the latter two reports. Organisms recovered from skin strips represented bacteria present in or on the skin of the incision site at the beginning of surgery. Wound swab cultures identified those bacteria which contaminated the wound from contiguous skin, airborne particles, operating room personnel, or fomites. Ordinary skin flora was frequently cultured from both types of specimens, but *Staph. aureus* was found in only nine skin strips, and in no instance did these strains cause wound sepsis in the corresponding patient. Later experience revealed a single instance of wound colonization and sepsis with a preoperative carrier strain which had been recovered from an operating room wound swab. These results suggest that, in the patients studied, colonization of the wound usually occurred postoperatively in the recovery room or on the ward rather than in the operating room. A similar conclusion was reported by Rountree *et al.* (1960).

The results of this study also indicate that colonization of the wound in a staphylococcal carrier is usually with the carrier strain. It remains for further investigation to indicate the route by which the carrier strain arrives in the wound and when contamination occurs. Prophylactic antibiotic administration might be helpful if the route is haematogenous, and mechanical exclusion of the wound from external contamination should be effective against organisms transferred by hands, fomites, and air. Rountree *et al.* (1960) were able to reduce wound sepsis significantly by sealing off the incision with an occlusive plastic spray dressing, although there were five instances of autogenous infection of the wound despite an intact seal.

According to the results of the present study, it should be possible to reduce the rate of wound sepsis by approximately one half by preventing or eliminating the carrier state preoperatively or by the use of an occlusive dressing. Many trials of various antibacterial creams, ointments, sprays and soaps for the control of the carrier state have yielded conflicting and equivocal results. In addition to the work of Rountree *et al.* mentioned above, we are investigating the use of an occlusive dressing ('Vidrape') for clean wounds. So far, only one instance of staphylococcal colonization has been observed in the wounds of 32 patients, five of whom were preoperative carriers.

SUMMARY

The relationship of pre-operative nasal and skin carriage of *Staphylococcus aureus* to wound colonization and sepsis was studied in 269 patients. Thirty-seven per cent of 96 carriers developed wound colonization as compared to 16 % among non-carriers (a statistically significant difference). The wound sepsis rates were 17 % and 9 % respectively. The combination of nasal and skin carriage was an important factor, since the sepsis rate among skin carriers (most of whom were

nasal carriers as well) was 22 %. Among carriers, the homologous strain was recovered from the majority of wound colonizations and from all instances of wound sepsis. A carrier strain also was recovered from 52 % of the wounds colonized and from 50 % of septic wounds in the entire study group. Profuse nasal carriage resulted in a significantly greater number of septic wounds (31 %) than sparse carriage (9 %). Wound cultures before closure, and skin from the initial incision site only once yielded a patient's carrier strain or a strain of *Staph. aureus* that was later recovered from the wound. The results indicate that measures designed to control the carrier state or to isolate the wound from the external environment should reduce wound sepsis by approximately one half.

Excellent technical aid was furnished by Eleanor Ford, R. N., Edith E. Silverman, Adrienne Marus, M. S., and Patricia Welch.

REFERENCES

ALTEMEIER, W. A., HUMMEL, R. P. & HILL, E. O. (1966). Prevention of infection in colon surgery. *Archs Surg., Chicago* **93**, 226.

BASSETT, H. F. M., FERGUSON, W. G., HUFFMAN, E., WALTER, M., BLOWERS, R. & CONN, C. A. (1963). Sources of staphylococcal infection in surgical wound sepsis. *J. Hyg., Camb.* **61**, 83.

BROWNE, A. F., RYAN, E. A., GLASSOW, F. J., MARTIN, C. J. & SHOULDICE, E. E. (1959). Staphylococcic wound infections: study of wound infections in several thousand hernia cases. *J. Am. med. Ass.* **170**, 1274.

BULLOCK, W. E., HALL, J. W., SPINK, W. W., DAMSKY, L. J., GREENE, V. W., VESLEY, D. & BAUER, H. (1964). A staphylococcal isolation service: epidemiologic and clinical studies over one year. *Ann. intern. Med.* **60**, 777.

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

BURKE, J F. & CORRIGAN, E. A. (1961). Staphylococcal epidemiology on surgical ward: fluctuations in ward staphylococcal contents, its effect on hospitalized patients and the extent of endemic hospital strains. *New Engl. J. Med.* **264**, 321.

COHEN, L. S., FEKETY, F. R. & CLUFF, L. E. (1964). Studies of the epidemiology of staphylococcal infection. VI. Infections in the surgical patient. *Ann. Surg.* **159**, 321.

COLBECK, J. C. (1960). Environmental aspects of staphylococcal infections acquired in hospitals. I. The hospital environment—its place in the hospital staphylococcus infections problems. *Am. J. publ. Hlth* **50**, 468.

COLE, W. R. & BERNARD, H. R. (1964). Relationship of skin carriage to post-operative staphylococcal wound infection. *Surg. Forum.* **15**, 52.

EHRENKRANZ, N. J. (1964). Person to person transmission of *Staphylococcus aureus*: quantitative characterization of nasal carriers spreading infection. *New Engl. J. Med.* **271**, 225.

FEKETY, F. R., JR. (1964). The epidemiology and prevention of staphylococcal infection. *Medicine, Baltimore* **43**, 593.

HOWE, C. W. & MARSTON, A. T. (1962). A study on sources of post-operative staphylococcal infections. *Surgery Gynec. Obstet.* **115**, 266.

JEFFREY, J. S. & SKLAROFF, S. A. (1958). Incidence of wound infection. *Lancet.* i, 365.

KETCHAM, A. S., BLOCH, J. H., CRAWFORD, D. I., LIEBERMAN, J. E. & SMITH, R. R. (1962). The role of prophylactic antibiotic therapy in control of staphylococcal infections following cancer surgery. *Surgery Gynec. Obstet.* **114**, 345.

LINDBOM, G. & LAURELL, G. (1967*a*). Studies on the epidemiology of staphylococcal infections. 4. Effect of nasal chemotherapy on carrier state in patients and on post-operative sepsis. *Acta. path. microbiol. scand.* **69**, 237.

LINDBOM, G. & LAURELL, G. (1967*b*). Studies on the epidemiology of staphylococcal infections. 5. Importance of environmental factors during the early post-operative phase. *Acta. path. microbiol. scand.* **69**, 246.

LINDBOM, G., LAURELL, G. & GRENVIK, A. (1967). Studies on the epidemiology of staphylococcal infections. 3. Influence of factors inherent in the patient and his operation. *Acta. path. microbiol. scand.* **69**, 219.

LOWDEN, T. G., VAITHILINGHAM, P. S. & MILNE, J. B. (1962). Post-operative infection in a casualty department. *Lancet* ii, 752.

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

MOORE, B. & GARDNER, A. M. N. (1963). A study of post-operative wound infection in a provincial general hospital. *J. Hyg., Camb.* **61**, 95.

ROBERTS, D. R. (1965). Significance of clean wound cultures. *Am. Surg.* **31**, 153.

ROGERS, L. S., DUFFY, J. P. & MOU, T. W. (1965). Staphylococcal sepsis and patient's nasal-carrier state. *Archs Surg., Chicago* **90**, 294.

ROUNTREE, P. M., HARRINGTON, M., LOEWENTHAL, J. & GYE, R. (1960). Staphylococcal wound infection in a surgical unit. *Lancet* ii, 1.

SHINEFIELD, H. R., RIBBLE, J. C., BORIS, M. & EICHENWALD, H. F. (1963). Bacterial interference: its effect on nursery-acquired infection with *Staphylococcus aureus*. I. Preliminary observations on artificial colonization of newborns. *Am. J. Dis. Child.* **105**, 646.

WALTER, C. W. (1958). Environmental sepsis. *Mod. Hosp.* **91**, 69.

WEINSTEIN, H. J. (1959). The relation between the nasal-staphylococcal-carrier state and the incidence of postoperative complications. *New Engl. J. Med.* **260**, 1303.

WHITE, A. (1961). Relation between quantitative nasal cultures and dissemination of staphylococci. *J. Lab. clin. Med.* **58**, 273.

WILLIAMS, R. E. O., JEVONS, M. P., SHOOTER, R. A., HUNTER, C. J. W., GIRLING, J. A., GRIFFITHS, J. D. & TAYLOR, G. W. (1959). Nasal staphylococci and sepsis in hospital patients. *Br. med. J.* ii, 658.

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.

WISE, R. I., SWEENEY, F. J., JR, HAUPT, G. J. & WADDELL, M. A. (1959). The environmental distribution of *Staphylococcus aureus* in an operating suite. *Ann. Surg.* **149**, 30.

J. Hyg., Camb. (1969), **67**, 59
Printed in Great Britain

Thermal requirements of secondary schoolchildren in winter

BY A. AULICIEMS
Department of Geography, University of Reading

(*Received* 18 *June* 1968)

INTRODUCTION

Although winter thermal comfort requirements in Great Britain have been investigated for adults in light industry (Bedford, 1936), offices (Black, 1954), and lecture halls (Angus & Brown, 1957), no inquiry appears to have been conducted with respect to schoolchildren.

Suggestions for desirable classroom temperatures have been largely based on considerations of the relatively higher metabolic rate of children and in consequence recommended temperatures have been appreciably lower than those considered optimum for adults. Clay (1929) advocated air temperatures of 55–60° F., Vernon, Bedford & Warner (1930) 'not much below 60° F.', and Seymour (1939) 55–57° F. equivalent temperature. However, Post War Building Studies No. 27, on heating and ventilating of schools (1947), suggested somewhat higher values with 57–60° F. equivalent temperature, while the Building Bulletin No. 2 (1950) specified an air temperature of 62° F. This is the present legal requirement (Statutary Instruments 1954), which stipulates 62° F. air temperature with six air changes per hour (20–40 ft./min. air movement).

Although the latter is regarded by the Institute of Heating and Ventilating Engineers (The Heating and Ventilation of Schools, 1951) as the minimum requirement, the reasons for and indeed the desirability of this temperature may be open to question since no experimental data have yet been presented. The present investigation was designed to determine the optimum conditions and limits for the thermal comfort of secondary schoolchildren.

METHOD

Data were collected from twenty-three classes of children drawn from nineteen secondary schools in the Reading area. The sample included Secondary Modern, Grammar and Public Schools from which 624 children (378 boys and 246 girls), aged approximately 11–16 years, acted as subjects.

Usual school clothing was worn and measurements were taken following tests of performance with the children seated as usual at their desks. Air temperature and humidity were measured by an Assmann psychrometer (a radiation-shielded dry- and wet-bulb thermometer combination aspirated by means of a clockwork or electric fan), thermal radiation was estimated by two 6 in. globe thermometers (an instrument in which the thermometer is inserted with the bulb at the centre of a blackened hollow copper sphere), and the rate of air movement was determined from a silvered Kata thermometer (this is a large-bulbed alcohol thermometer

which is heated by immersion in hot water, dried and allowed to cool: the time required for cooling over a final temperature range is proportional to the air velocity). The instruments were located in two central positions on desks at sitting head height, readings were averaged and equivalent temperature (T_{eq}) and corrected effective temperature (C.E.T.) determined. These indices facilitate description and assessment of the thermal environment since they combine several atmospheric factors into *single* numerical values, which are conveniently derived by reference to charts. T_{eq} integrates air temperature, radiation and air velocity and C.E.T. combines radiation, humidity and air velocity. (For further description of instruments and indices see Macpherson, 1962.)

Subjective impressions of warmth were recorded on the seven-point scale as used by Bedford (1936). The scale was printed on the blackboard and the children were asked to consider individually their sensations and enter the corresponding numeral in appropriate spaces on their performance test papers. The arbitrary units 1–7 were considered to be the most simple for the children, but for statistical analysis were altered to those as shown in Table 1.

Table 1. *Scale of thermal sensations*

Sensation	Numerical value used by children	Numerical value used in analysis
Much too warm	1	+3
Too warm	2	+2
Comfortably warm	3	+1
Comfortable	4	0
Comfortably cool	5	−1
Too cool	6	−2
Much too cool	7	−3

Most assessments were made 40–70 min. after the children had entered the room, but on seven occasions after 25–30 min. The data relate to both the mornings and afternoons between January and April 1967, and October 1967 and March 1968. Thermal conditions were altered occasionally in an attempt to lower excessive temperatures, but otherwise left as found by the investigator. It is believed that the information gained is very representative of classroom conditions of normal occupancy and activity.

RESULTS

The frequency of thermal conditions encountered appears in Table 2 in terms of four thermal measures, and the distribution of thermal sensations appears in Table 3. In general, ventilation rates were very low and on fifty-three sessions the rate of air movement was less than 10 ft./min., while 20 ft./min. was exceeded on only nineteen occasions.

Although the scale of numerical values is quite arbitrary, linear relationships between mean values and thermal conditions have been established by previous investigators. Tables 4 and 5 show the distribution of thermal sensations and corresponding means for intervals of air temperature and T_{eq}.

Graphical representation of the means against equivalent temperature is shown

Table 2. *Distribution of thermal conditions*

Intervals (°F.)	Frequency			
	Dry bulb	Globe	T_{eq}	C.E.T.
76·5–78·4	2	.	.	.
74·5–76·4	4	7	2	.
72·5–74·4	9	7	5	.
70·5–72·4	11	22	12	3
68·5–70·4	22	20	18	3
66·5–68·4	22	23	18	23
64·5–66·4	14	11	21	26
62·5–64·4	9	10	13	28
60·5–62·4	8	5	9	13
58·5–60·4	5	3	6	9
56·5–58·4	1	1	2	1
54·5–56·4	1	.	2	3
52·5–54·4	1	.	1	.
Total	109	109	109	109

Table 3. *Distribution of thermal sensations*

Sensation	Numerical value	Frequency		
		Boys	Girls	Total
Much too warm	+3	97	132	229
Too warm	+2	272	304	576
Comfortably warm	+1	308	312	620
Comfortable	0	288	249	537
Comfortably cool	−1	234	214	448
Too cool	−2	77	95	172
Much too cool	−3	21	21	42
Total		1297	1327	2624

Table 4. *Distribution of sensations in relation to dry-bulb temperature*

Dry bulb (°F.)	Frequency							Total	Mean sensation
	+3	+2	+1	0	−1	−2	−3		
76·5–78·4	19	21	9	4	1	1	.	55	+1·91
74·5–76·4	10	32	51	21	21	5	.	140	+0·81
72·5–74·4	24	72	43	36	15	.	.	190	+1·28
70·5–72·4	66	103	66	42	22	4	1	304	+1·44
68·5–70·4	24	114	155	127	83	20	3	526	+0·61
66·5–68·4	40	107	115	106	78	21	4	471	+0·67
64·5–66·4	35	76	71	64	63	17	8	334	+0·62
62·5–64·4	4	21	39	50	69	38	10	231	−0·35
60·5–62·4	6	15	53	54	51	27	6	212	−0·10
58·5–60·4	1	13	11	22	25	18	5	95	−0·38
56·5–58·4	.	.	1	1	7	8	3	20	−1·55
54·5–56·4	.	2	6	9	4	5	1	27	−0·26
52·5–54·4	.	.	.	1	9	8	1	19	−1·47
Total	229	576	620	537	448	172	42	2624	

in Fig. 1. Inspection reveals linearity, and the abrupt increase of slope at the upper end, as found by Hickish (1955) for adults in summer, is not readily evident. Regression analysis has been applied to individual scores and regression equations

Table 5. *Distribution of sensations in relation to equivalent temperature*

T_{eq} (°F.)	Frequency +3	+2	+1	0	−1	−2	−3	Total	Mean sensation
74·5–76·4	18	19	18	6	2	.	.	63	+1·71
72·5–74·4	19	52	40	24	9	.	.	144	+1·33
70·5–72·4	26	88	66	55	27	6	.	268	+1·05
68·5–70·4	66	114	129	75	48	9	1	442	+1·10
66·5–68·4	20	100	122	105	80	20	3	450	+0·56
64·5–66·4	48	109	105	100	92	27	5	486	+0·63
62·5–64·4	24	48	51	70	69	41	11	314	+0·11
60·5–62·4	2	19	42	46	52	24	9	194	−0·21
58·5–60·4	6	20	36	38	38	18	3	159	+0·07
56·5–58·4	.	5	4	7	11	6	5	38	−0·63
54·5–56·4	.	2	7	10	11	13	4	47	−0·81
52·5–54·4	.	.	.	1	9	8	1	19	−1·47
Total	229	576	620	537	448	172	42	2624	

Table 6. *Regression constants, correlation coefficients and optimum conditions*

Thermal measure (°F.)	r	Constant a	Constant b	Optimum condition
Dry bulb	0·37	0·116	−7·273	62·7
Globe	0·32	0·120	−7·643	63·7
T_{eq}	0·36	0·119	−7·341	61·7
C.E.T.	0·36	0·166	−10·097	60·8

Table 7. *Limits of the comfort zone for children*

Thermal measure (°F.)	Lower limit	Upper limit
Dry bulb	59 (58·5)	70 (70·4)
Globe	61 (60·5)	70 (70·4)
T_{eq}	59 (58·5)	68 (68·4)
C.E.T.	57 (56·5)	66 (66·4)

expressed in the form of $Y = ax+b$, where Y is thermal sensation, a and b constants and x thermal measure. Optimum values of thermal conditions, or those producing a thermal sensation of 0 (or 'comfortable') have been calculated and appear in Table 6, together with the regression constants and the product moment correlation coefficient (r). As pointed out by Hickish (1955), although the mean sensation of a group may be predicted with reasonable accuracy, prediction of an individual's sensation is highly unreliable.

To determine acceptable upper and lower limits for comfort the data have been classified into three categories within which the children may be considered as

feeling comfortable (values +1, 0, −1), too warm (values +3, +2) and too cool (values −2, −3). Percentages were determined for the three categories for each interval of temperature and the percentage comfortable appears in Fig. 2 in relation to T_{eq}.

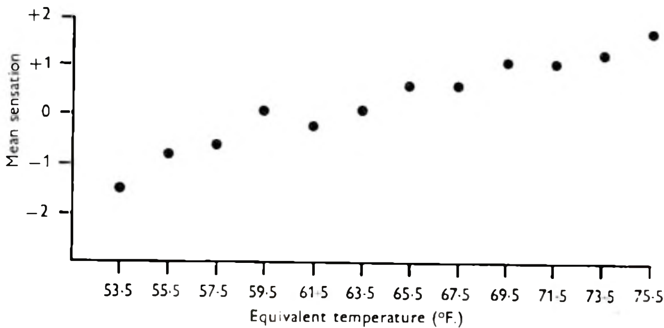


Fig. 1. Mean sensations against equivalent temperature.

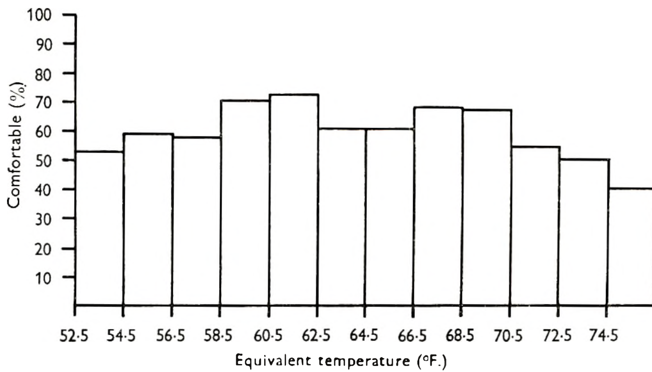


Fig. 2. Percentage of children comfortable in relation to equivalent temperature.

The delineation of the comfort zone is arbitrary and limits have varied greatly with investigators. Hickish (1955) selected a comfort zone on an 80% comfortable basis, Bedford (1936) chose 70% while Partridge & MacLean (1935) and Houghten & Yagloglou (1923) used only 50%. In the present study over 70% comfort conditions occurred very rarely and no comfort zone could be established on this basis. However, a choice of temperatures within which 60% of the children were comfortable seemed satisfactory, and it is this percentage which has been selected. The upper and lower delineation is shown in Table 7.

DISCUSSION

Since conditions of still air were mainly encountered in these tests, the difference in physiologic effect between the optimum air temperature at 62·7° F. and the specified 62° F. (Statutary Instruments, 1954) with its greater air movement becomes appreciably magnified. The latter figure would probably approximate

59° F. T_{eq}, which is nearly three degrees below the optimum found at 61·7° F. T_{eq}. Alternatively, given conditions of approximately equal temperatures of surroundings and air, to produce this optimum T_{eq} with air movement of 30 ft./min., air temperature of approximately 65° F., would be required.

Bedford (1936) found that optimum conditions for adults at sedentary work were at approximately 62° F. T_{eq}, or 65° F. air temperature with average air motion of 30 ft./min. More recently Black (1954) revealed somewhat higher values at 66–67° F. air temperature, but regrettably failed to measure rates of air movement. In comparison, it would seem that if the required rates of ventilation were maintained in schools, the optimum for children in terms of dry-bulb temperature would lie only slightly below those of adults. This finding is supported by the earlier study of Partridge & MacLean (1935), who found that the thermal requirements of children in Canada were almost identical with those of adults. The raised thermal neutrality of children can possibly be explained by acclimatization due to the habitual exposure of children to adult-regulated micro-climates.

The present study indicates that the difficulty in schools is one of overheating rather than that of achieving sufficiently high temperatures. This appears to be associated with the failure to maintain adequate ventilation rates, a problem also observed in classrooms by Weston (1953), and a partial solution may be found by wider window opening.

SUMMARY

Secondary schoolchildren in England were asked to assess thermal conditions in winter according to subjective sensations of warmth. Conditions of normal occupancy and activity were maintained and optimum conditions established in terms of four thermal measures by regression analysis of over 2500 assessments by boys and girls. The comfort zone was delineated as that within which 60 % of the children were comfortable and in terms of the commonly used dry-bulb temperature this zone lay between 59 and 70° F. in still air. The optimum conditions for children were also compared with those recommended for adults.

REFERENCES

ANGUS, T. C. & BROWN, J. R. (1957). Thermal comfort in the lecture room. An experimental study of winter requirements. *J. Instn Heat. Vent. Engrs* **25**, 175.

BEDFORD, T. (1936). The warmth factor in comfort at work. *Rep. ind. Hlth Res. Bd*, no. 76.

BLACK, F. (1954). Desirable temperatures in offices. A study of occupant reaction to heating provided. *J. Instn Heat. Vent. Engrs* **22**, 319.

BUILDING BULLETIN NO. 2 (1950). Ministry of Education. H.M.S.O.

CLAY, F. (1929). *Modern School Buildings*. London: Batesford.

HICKISH, D. E. (1955). Thermal sensations of workers in light industry in summer. A field study in Southern England. *J. Hyg., Camb.* **53**, 112.

HOUGHTEN, F. C. & YAGLOGLOU, C. P. (1923). Determination of the comfort zone. *Trans. Am. Soc. Heat. Vent. Engrs* **29**, 361.

MACPHERSON, R. K. (1962). The assessment of the thermal environment. A review. *Br. J. ind. Med.* **19**, 151.

PARTRIDGE, R. C. & MACLEAN, D. L. (1935). Determination of the comfort zone for school children. *J. ind. Hyg.* **17**, 66.

POST WAR BUILDING STUDIES NO. 27 (1947). *Heating and Ventilation of Schools*. H.M.S.O.

SEYMOUR, D. W. (1939). *Heating, Ventilation and Lighting of School Buildinas*. Oxford University Press.

STATUTARY INSTRUMENTS NO. 473 (1954). Statutary Publications Office.

THE HEATING AND VENTILATION OF SCHOOLS (1951). *J. Instn Heat. Vent. Engrs* **18**, 466.

VERNON, H. M., BEDFORD, T. & WARNER, C. G. (1930). A study of heating and ventilation in schools. *Rep. ind. Hlth Res. Bd.* no. 58.

WESTON, J. C. (1953). School heating research. *J. Instn Heat. Vent. Engrs* **20**, 459.

J. Hyg., Camb. (1969), **67**, 67
Printed in Great Britain

HI antibody to various influenza viruses and adenoviruses in individuals of blood groups A and O

BY C. W. POTTER
Department of Medical Microbiology and Virus Research Laboratories, University of Sheffield

(*Received* 22 *June* 1968)

INTRODUCTION

The relationship between blood group and the incidence of certain virus infections in man has been studied by several workers (reviewed by Allison, 1965). A study of servicemen admitted to sick-quarters with acute respiratory infections during the period 1956–61 indicated that infection by A 2 influenza virus was more common in persons of blood group O than in persons of blood group A (McDonald & Zuckerman, 1962). In addition, antibody to A 2 influenza virus was found in a significantly higher proportion of serum specimens from civilian persons of blood group O than from similar aged persons of blood group A (Potter & Schild, 1967). These independent studies suggest that susceptibility of man to infection by A 2 influenza virus is not uniform, and that the variability is genetically linked to blood-group status.

To extend these observations on the susceptibility of persons of blood groups A and O to A 2 influenza virus infection, the incidence of antibody to representative A_0, A, A_1 and A_2 influenza viruses was carried out in serum specimens collected in 1966. A study of antibody to adenovirus types 3 and 7 was also performed as McDonald & Zuckerman (1962) observed a significantly higher infection rate by adenovirus for servicemen of blood group A as compared with blood group O.

MATERIALS AND METHODS

Serum specimens

Serum specimens were obtained during the period April–October 1966 from 369 persons of known age and blood group A or O. The majority of the serum specimens were from Sheffield blood donors. The remainder, and all those from individuals under 18 years of age, were specimens submitted to the Sheffield Children's Hospital for antistreptolysin 'O' testing.

Serum from a further 382 persons of blood group A or O and of known age were examined for antibody to adenovirus. These specimens were collected in 1961–3 from children admitted to the Sheffield Children's Hospital for treatment of trauma or accidents or from children with a confirmed diagnosis of appendicitis. Specimens from older age groups were from 150 women attending antenatal clinics and from blood donors.

All sera were from persons living in the Sheffield region.

Viruses

A/Swine (Shope Sw. 15, 1930), A/PR 8 (1934), A 1/FM 1 (1947) and A 2/Singapore/1/57 were stock strains adapted to growth in the allantoic cavity. Virus pools were prepared by allantoic inoculation of 10-day fertile fowl eggs with 10^{-3} or 10^{-4} dilutions of seed virus. After incubation at 35° C. for 48 hr., allantoic fluids were harvested, pooled and stored in sealed ampoules at −70° C.

Adenovirus types 3 and 7 were cultured on HeLa cells maintained in Eagle's basal medium with 2 % inactivated calf serum, 0·88 g./l. sodium bicarbonate and antibiotics. When complete cytopathic effect was observed, cultures were frozen to −70° C. and thawed twice, centrifuged at 3000 rev./min. for 30 min. and the supernatant fluids stored in sealed ampoules at −70° C.

Haemagglutination inhibition (HI) tests

Serological tests for HI antibody to influenza viruses were carried out by standard methods in Perspex plates (W.H.O., 1953) using eight haemagglutinating units of virus (50 % end-point). Before testing, serum specimens were incubated for 18 hr. at 37° C. with 5 volumes of cholera filtrate (N. V. Philips Duphar, Amsterdam) and subsequently heated at 56° C. for 1 hr. Fowl erythrocytes (0·5 % suspension in phosphate buffered saline (PBS)) were used.

Serum specimens tested for HI antibody to adenovirus types 3 and 7 were pretreated by the methods described by Rosen (1960). HI tests were carried out by incubating 0·2 ml. of virus, containing four haemagglutinating units, for 1 hr. at room temperature with an equal volume of pretreated serum dilution. After this time, 0·2 ml. of 1 % Patas monkey cells were added and the tests were further incubated at 37° C. for 1 hr. HI antibody titres were taken as the highest serum dilution which gave 50 % inhibition of virus haemagglutination.

RESULTS

Incidence of HI antibody in serum specimens to influenza viruses

The overall incidence of HI antibody at 1/6 or greater serum dilution to four influenza viruses is given in Fig. 1. The proportions of serum specimens with antibody at various ages differed with the various strains. HI antibody to A 2/Singapore/1/57 was detected in 224 of 369 specimens (61 %) from persons aged 11–60 years; the highest incidence was found in persons aged 16–20 years (91 %) and the lowest in persons aged 51–60 years (29 %). HI antibody to A 1/FM 1, A/PR 8 and A/Swine was found in 142 (38 %), 136 (37 %) and 104 (28 %) of serum specimens, respectively. Antibody to A 1/FM 1 virus was not found in serum specimens from persons aged less than 16 years and the highest incidence (82 %) was found in serum specimens from persons aged 21–25 years. HI antibody to A/PR 8 and A/Swine was not detected in persons aged less than 21 years and 26 years, respectively. The highest incidence for HI antibody to A/PR 8 virus was found in persons aged 26–30 years (86 %) and to A/Swine virus in persons aged 51–60 years (91 %).

This age distribution of HI antibody to influenza viruses was very similar to that reported by other workers (Schild & Stuart-Harris, 1965).

HI antibody to influenza virus in serum specimens from persons of blood groups A and O

The sera tested included 209 specimens from persons of blood group O and 160 specimens from persons of blood group A. The incidence of HI antibody at

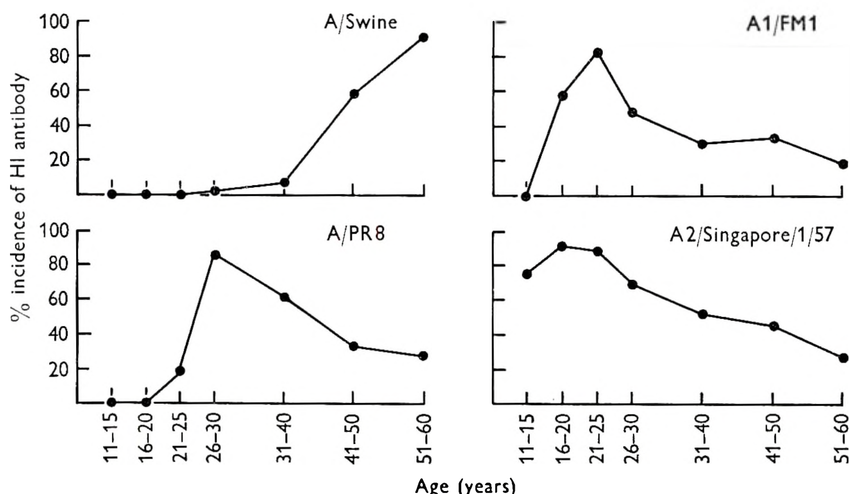


Fig. 1. Percentage incidence of HI antibody to four influenza viruses in serum specimens from individuals of various ages.

Table 1. *Incidence of HI antibody in serum specimens from persons of blood groups A and O to four influenza viruses*

Age (years)	Blood group	No. with HI antibody to A/swine	A/PR8	A1/FM_1	A2/Sing/1/57
11–15	O	0/18*	0/18	0/18	14/18 (78)
	A	0/11	0/11	0/11	8/11 (73)
16–20	O	0/18	0/18	10/18 (56)	16/18 (89)
	A	0/17	0/17	10/17 (59)	16/17 (94)
21–25	O	0/31	7/31 (23)	23/31 (74)	28/31 (90)
	A	0/25	3/25 (12)	23/25 (92)	22/25 (88)
26–30	O	0/30	26/30 (87)	15/30 (50)	21/30 (70)
	A	1/19 (5)	16/19 (84)	8/19 (42)	13/19 (68)
31–40	O	5/45 (11)	30/45 (67)	13/45 (29)	23/45 (51)
	A	0/25	13/25 (52)	8/25 (32)	14/25 (56)
41–50	O	25/40 (62·5)	13/40 (32·5)	14/40 (35)	16/40 (40)
	A	13/24 (54)	9/24 (37·5)	7/24 (29)	14/24 (58)
51–60	O	24/27 (89)	10/27 (37)	2/27 (7)	7/27 (26)
	A	36/39 (92)	9/39 (23)	10/39 (26)	12/39 (31)

* $\frac{\text{No. positive}}{\text{No. tested}}$. Figures in parentheses indicate percentage positive.

1/6 or greater serum dilution to each of four influenza viruses is given for persons of blood groups O and A separately and in seven age groups in Table 1. The findings are shown in percentages in Fig. 2. HI antibody to A/Swine virus was detected in four of the seven age groups; in two groups, aged 31–40 years and 41–50 years, a higher percentage of serum specimens from persons of blood group O contained antibody compared with persons of blood group A whilst the reverse was found in the age groups 26–30 years and 51–60 years. HI antibody to A/PR 8 virus was present in five of the seven age groups tested; a higher proportion of antibody positive sera from persons of blood group O, compared with persons of blood

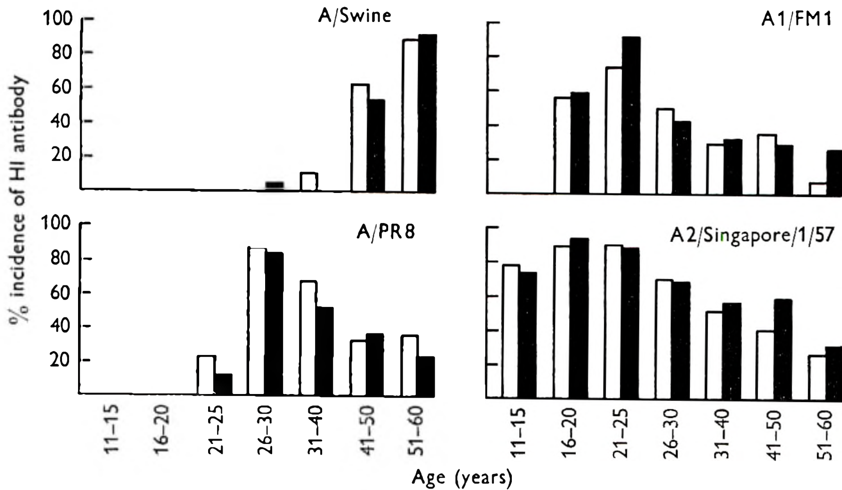


Fig. 2. Percentage incidence of HI antibody to influenza viruses in serum specimens from individuals of blood groups A and O of various ages. □, Blood group O; ■, blood group A.

group A, was found in four of the five groups. HI antibody to A/FM1 and A/Singapore/1/57 was found in a greater proportion of persons of blood group O compared with persons of blood group A, in two of six and three of seven of the age groups, respectively. None of these differences, either individually or collectively, were significant (χ^2 test (Yates correction); $P = > 0{\cdot}05$).

HI antibody in serum specimens to adenovirus types 3 *and* 7

HI antibody titres to adenovirus types 3 and 7 were determined for each of 382 serum specimens from individuals of blood groups A and O. The incidence of HI antibody to adenovirus type 3 at 1/10 dilution or greater (Table 2) rose from 30 % in children aged 1–5 years to a maximum of 62 % for persons aged 21–25 years. HI antibody to adenovirus type 7 was detected in 7 % of serum specimens from persons aged 1–5 years and increased to a maximum of 41·5 % in persons aged 26–30 years.

Figure 3 shows the incidence of HI antibody to adenovirus types 3 and 7 in persons of blood group A and O separately. The incidence of antibody to adenovirus type 3 was greater in persons of blood group O than in persons of blood group A in

the three youngest age groups; for persons aged 16 years and older, the incidence of antibody in persons of blood group O was essentially the same as in persons of blood group A. HI antibody to adenovirus type 7 was found in a higher proportion of persons of blood group O aged 1–25 years than in similarly aged persons of blood group A. For persons aged 26 years and over, the incidence of HI antibody to adenovirus type 7 was the same for persons of blood groups A and O.

Table 2. *Incidence of HI antibody to adenovirus types* 3 *and* 7 *in serum specimens from individuals of various ages*

Age (years)	No. tested	HI antibody to Adenovirus-3	HI antibody to Adenovirus-7
1–5	30	9 (30 %)	2 (7 %)
6–10	65	24 (37 %)	9 (14 %)
11–15	54	22 (41 %)	12 (22 %)
16–20	51	25 (49 %)	13 (23·5 %)
21–25	60	37 (62 %)	21 (34 %)
26–30	53	30 (57 %)	22 (41·5 %)
31–40	52	25 (48 %)	21 (40 %)
41–50	17	7 (41 %)	5 (29 %)

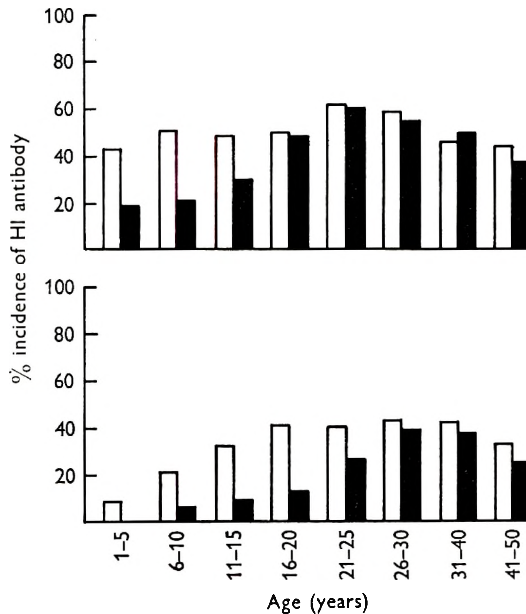


Fig. 3. Percentage incidence of HI antibody to adenovirus types 3 and 7 in serum specimens from individuals of blood group A and O of various ages. □, Blood group O; ■, blood group A.

The significance of the variable distribution of HI antibody to adenovirus types 3 and 7 for persons of blood groups A and O is shown in Tables 3 and 4. For persons aged 1–15 years HI antibody was detected in a significantly higher proportion of persons of blood group O, compared with persons of blood group A. Only in the

age group 6–10 years was the difference significant for an individual age group. HI antibody to adenovirus type 7 was detected in a significantly greater number of persons of blood group O, compared with persons of blood group A, for individuals aged less than 21 years. However, in no individual age group was the difference significant.

Table 3. *Distribution of HI antibody to adenovirus type* 3 *in sera from individuals of blood group A and O*

Age (years)	Blood group	No. tested	No. positive	No. negative	χ^2 (Yates correction)
		HI antibody to Adenovirus type 3			
1–5	O	14	6 (4·2)	8 (9·8)	1·078
	A	16	3 (4·8)	13 (11·2)	
6–10	O	33	17 (12·2)	16 (20·8)	4·887*
	A	32	7 (11·8)	25 (20·2)	
11–15	O	31	15 (12·6)	16 (18·4)	1·1323
	A	23	7 (9·4)	16 (13·6)	

Figures in parentheses indicate expected values.
* $P = < 0{\cdot}05$.
Total χ^2 values (Yates correction). $\chi^2 = 7{\cdot}0973$; $n = 3$; $P = < 0{\cdot}05$.

Table 4. *Distribution of HI antibody to adenovirus type* 7 *in sera from individuals of blood groups A and O*

Age (years)	Blood group	No. tested	No. positive	No. negative	χ^2 (Yates correction)
		HI antibody to Adenovirus type 7			
1–5	O	14	2 (1·0)	12 (13·0)	2·1436
	A	16	0 (1·0)	16 (15·0)	
6–10	O	33	7 (4·6)	26 (28·4)	1·8631
	A	32	2 (4·4)	30 (27·6)	
11–15	O	31	10 (7·0)	21 (24·0)	2·7506
	A	23	2 (5·0)	21 (18·0)	
16–20	O	22	9 (5·6)	13 (16·4)	3·5412
	A	29	4 (7·4)	25 (21·6)	
21–25	O	37	15 (13·0)	22 (24·0)	0·6982
	A	23	6 (8·0)	17 (15·0)	

Figures in parentheses indicate expected values for age groups 1–20 years. χ^2 (Yates correction) $= 10{\cdot}2985$; $n = 4$; $P = < 0{\cdot}05$.

DISCUSSION

Influenza A2 virus first appeared in 1957; at this time the entire population was immunologically susceptible to infection (Mulder, 1957). A study of serum specimens collected in the period 1961–3, after the first few years of prevalence of A2 influenza virus, indicated that infection had occurred in a greater proportion of persons of blood group O than of persons of blood group A (Potter & Schild, 1967). These findings were consistent with those of McDonald & Zuckerman (1962). The

present study of serum specimens collected in 1966, from the same population but one exposed to further waves of infection by A 2 influenza virus, indicated that past infection had occurred with the same frequency in persons of blood group A and O. In addition, evidence of past infection by A/Swine, A/PR 8 and A 1/FM 1 was found with the same frequency for persons of the two blood groups.

The changes in antibody distribution for influenza A 2 virus with respect to blood groups may be due to changes in the immune status of the population following successive waves of infection. Infections in the first waves of A 2 influenza would occur predominantly in the more susceptible blood group O persons. Later waves of infection would occur in a heterogeneous population with certain individuals possessing, as the result of past infection, protective antibody and others being antibody-negative. The incidence of influenza in less susceptible, blood group A, persons would increase with successive waves of infection as blood-group status does not relate to absolute resistance. Thus, repeated exposure of a population to epidemics of A 2 virus influenza would result in obscuring or obliterating genetically determined degrees of susceptibility.

HI antibody to adenovirus types 3 and 7, two of the three adenovirus serotypes most prevalent in military populations (Hilleman *et al.* 1957; McDonald *et al.* 1958), was detected in a higher proportion of persons of blood group O than in persons of blood group A in the younger age groups only. In older age groups the incidence of antibody was the same for persons of blood groups A and O.

The explanation suggested to account for the changes in antibody status for influenza viruses may be extended to the studies with adenoviruses. In the younger age groups infections by adenovirus types 3 and 7, as indicated by the presence of HI antibody, occur in a significantly greater proportion of persons of blood group O than persons of blood group A. From the results of the present study, this is seen for the age groups 1–10 years for adenovirus type 3 and age 1–20 years for adenovirus type 7. After these ages adenovirus infections will occur with relatively greater frequency in antibody negative, blood group A persons. This conclusion is indicated as in older age groups serological evidence of part infection is found with equal frequency in persons of both blood groups. The ages when infections by adenovirus types 3 and 7 occur more frequently in blood group A persons, compared with persons of blood group O, include a high proportion of military personnel, and it was in such a group that McDonald & Zuckerman (1962) reported a higher infection rate by adenovirus for persons of blood group A compared with those of blood group O.

SUMMARY

Serum specimens collected in 1966 from individuals of different age groups were studied for the presence of haemagglutination inhibition (HI) antibody to four influenza viruses. All sera were from individuals of blood group A or O and in no instance was the incidence of antibody to a virus strain significantly greater for persons of blood group O compared with similar aged individuals of blood group A. This finding for HI antibody to A 2/Singapore/1/57 is different from similar studies of serum specimens collected in 1961–3. It is suggested that the change in the

immune status of a population, with reference to blood-group status, is due to repeated exposure to infection; this changing pattern of immune status is discussed.

Similar studies of HI antibody to adenovirus type 3 and 7 in human sera from persons of blood group A and O shows a changing pattern with increasing age. These results are consistent with the findings for influenza virus and are discussed. Repeated exposure of a population to infection results in obscuring genetically determined variations in susceptibility.

I would like to thank Professor M. G. McEntegart, Professor of Medical Microbiology, and Professor C. H. Stuart-Harris, Professor of Medicine, for their advice and criticism, and Miss D. Coles, F.I.M.L.T., and Mrs P. Robinson for expert technical assistance.

REFERENCES

ALLISON, A. C. (1965). Genetic factors in resistance against virus infections. *Arch. ges. Virusforsch.* **17**, 280.

HILLEMAN, M. R., GAULD, R. L., BUTLER, R. L., STALLONES, R. A., HEDBERG, C. L., WARFIELD, M. S. & ANDERSON, S. A. (1957). Appraisal of occurrence of adenovirus-caused respiratory illness in military populations. *Am. J. Hyg.* **66**, 29.

MCDONALD, J. C., WILSON, J. S., THORBURN, W. B., HOLLAND, W. W. & ANDREWS, B. E. (1958). Acute respiratory disease in the R.A.F., 1955–7. *Br. med. J.* ii, 721.

MCDONALD, J. C. & ZUCKERMAN, A. J. (1962). ABO blood groups and acute respiratory virus disease. *Br. med. J.* ii, 89.

MULDER, J. (1957). Asian influenza in the Netherlands. *Lancet.* ii, 334.

POTTER, C. W. & SCHILD, G. C. (1967). The incidence of HI antibody to influenza virus A2/Singapore/1/57 in individuals of blood groups A and O. *J. Immun.* **98**, 1320.

ROSEN, L. (1960). A haemagglutination-inhibition technique for typing adenoviruses. *Am. J. Hyg.* **71**, 120.

SCHILD, G. C. & STUART-HARRIS, C. H. (1965). Serological epidemiological studies with influenza A viruses. *J. Hyg., Camb.* **63**, 479.

WORLD HEALTH ORGANIZATION (1953). Expert Committee on Influenza, *Tech. Rep. Ser. Wld Hlth Org.* no. 64.

J. Hyg., Camb. (1969), **67**, 75
Printed in Great Britain

Salmonella senftenberg in the Sunderland area

BY D. A. SANFORD,* D. A. LESLIE* AND J. A. McKEON*
Ryhope Hospital, Sunderland

P. B. CRONE*
Public Health Laboratory, Sunderland

AND BETTY C. HOBBS
Food Hygiene Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9

(*Received* 24 *July* 1968)

In December 1963 a patient was found to be excreting *Salmonella senftenberg* in general hospital A (Ryhope) of 300 beds. From then onwards about 150 staff and patients of this hospital excreted the organism. In 19 of the 48 months from December 1963 to November 1967 at least one excreter was found. The longest spell free of one was 7 months; usually the clear spells were only 2 or 3 months. The experience during this time of the surrounding area was different both in other hospitals (two isolations from a population more than 5 times as great), in general practice (three isolations all connected with hospital A in a population less frequently sampled) and in public health (no isolations).

Salmonella senftenberg *isolations in the hospital*

The isolations are shown in Table 1.

By early 1965 one of us (D.A.L.) carrying out the bacteriology had noted the undue numbers of excreters. He was unable, however, to establish a clear connexion between them. In April some patients on ward 6 had mild intestinal upsets. They were examined and the staff of the ward and that of the general kitchen were examined also. *S. senftenberg* was isolated from two on this ward and one on another ward, but not from the kitchen staff. In May more mild illness led to an investigation of patients and staff on ward 12 and in the theatre. Many excreters were found. A connexion between the theatre and ward 12 seemed established and the outbreak appeared to be localized. Very soon, however, it was clear that this was not so and excreters were found in another ward and among the administrative staff. In June they were found in most departments of the hospital. Among the catering staff twelve excreters were now discovered. The regular staff of the pathology and radiology departments escaped completely; however, to the former was attached a cadet nurse and to the latter a temporary typist. These two alone of those employed in the two departments ate in the canteen and both excreted *S. senften-*

* Members of the Control of Infection Committee, Ryhope Hospital, Sunderland. Requests for reprints should be addressed to Dr B. C. Hobbs, Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9.

Table 1. *Isolations of* Salmonella senftenberg *at hospital A and elsewhere in the Sunderland area*

	Month	Hospital A: Patients	Ward staff	Catering staff	Other staff	Total	Elsewhere
1963	Dec.	2 (5, 7)	—	—	—	2	—
1964	Jan.	2 (7, 17)	—	—	—	2	—
	Feb.	—	—	—	—	—	—
	Mar.	—	—	—	—	—	—
	Apr.	—	—	—	—	—	—
	May	—	—	—	—	—	—
	Jun.	1 (6)	1 (6)	—	—	2	—
	Jul.	—	—	—	—	—	—
	Aug.	—	—	—	—	—	—
	Sept.	—	—	—	—	—	—
	Oct.	—	—	—	—	—	—
	Nov.	1 (3)	—	—	—	1	—
	Dec.	1 (7)	1 (2)	—	—	2	—
1965	Jan.	—	—	—	—	—	—
	Feb.	—	—	—	—	—	—
	Mar.	—	—	—	—	—	—
	Apr.	2 (6, 11)	1 (6)	—	—	3	—
	May	28 (6, 7, 12)	14 (6, 12)	—	10	52	—
	Jun.	30*	20†	12	17	79	—
	Jul.	—	—	—	—	—	—
	Aug.	—	—	1	—	1	—
	Sept.	—	—	1	—	1	—
	Oct.	—	—	1	—	1	—
	Nov.	—	—	—	—	—	—
	Dec.	1 (11)	—	3	—	4	—
1966	Jan.	—	—	2	1	3	—
	Feb.	1 (12)	—	—	—	1	2‡
	Mar.	—	—	—	—	—	—
	Apr.	—	—	—	—	—	—
	May	—	—	—	—	—	—
	Jun.	—	—	3	—	3	—
	Jul.	1 (12)	—	—	—	1	1§
	Aug.	1 (3)	—	1	—	2	—
	Sept.	—	—	—	—	—	1‖
	Oct.	—	—	—	—	—	—
	Nov.	—	—	—	—	—	—
	Dec.	—	—	—	—	—	—
1967	Jan.	—	—	—	—	—	—
	Feb.	—	—	—	—	—	—
	Mar.	—	—	—	—	—	1¶
	Apr.	1 (18)	—	—	—	1	—
	May	—	—	—	—	—	—
	Jun.	—	—	—	—	—	—
	Jul.	5 (10)	1 (10)	—	1	7	2**
	Aug.	—	—	—	—	—	—
	Sept.	—	—	—	—	—	—
	Oct.	—	—	—	—	—	—
	Nov.	—	—	—	—	—	—

Figures in parentheses indicate ward numbers.
* Wards 2, 4–7, 11, 12, 14 and 18.
† Wards 2–5, 7, 11, 14, 17 and 18.
‡ Mother and baby at hospital B.
§ Husband of one of staff of hospital A
‖ From septic tank at home of one of staff of Hospital A.
¶ Child in Hospital C.
** Relatives of Staff at Hospital A.

berg. The finding of these two excreters strengthened the view that such widespread involvement of the hospital could only result from dissemination by the kitchen. The hospital was closed as far as possible, and was gradually reopened throughout July, the last ward being opened in August. The kitchen meanwhile was closed on 5 June and reopened on 5 July. During this period food was brought in from outside.

About four-fifths of the patients and staff excreting the organism in May and June, and indeed at all times since December 1963, were wholly without symptoms. Most of the remainder were mildly ill. There were no deaths attributable solely to the infection but it is possible that the lives of a few very ill patients were shortened.

Throughout the rest of 1965 excreters continued to be detected in the catering staff. A single patient was also found to be excreting the organism. This pattern of detection continued until August 1966 but from then until April 1967 no more excreters were found. This was in spite of the continuing examination of the catering staff. During this period a comparable number of samples was obtained from other staff. None yielded *S. senftenberg.*

In July 1967 there was a party on ward 10 and some illness in patients, but samples were negative; 12 days later seven excreters of *S. senftenberg* were discovered. One of them was the ward maid, another a corridor cleaner friendly with her, both were ill.

Salmonella senftenberg *isolations elsewhere*

Isolations other than in the hospital are shown in Table 1 for the period since December 1963. Before that time *S. senftenberg* had been isolated in the Sunderland area in 1952 from an excreter of *S. paratyphi B*. It had been isolated from animal feeding stuff from a factory about 3 miles north of the hospital in October 1962 and June 1963. The factory had supplied feeding stuffs to a farm very near and towards the south-west of the kitchen hut of the hospital, but did not do so after December 1963. A mother and her baby excreted the organism during her lying-in period in February 1966 in hospital B. The obstetricians at the hospital are on the staff of hospital A. Neither was excreting *S. senftenberg.* In March 1967 a child in hospital C excreted the organism. No connexion could be established with hospital A. He lived in Boldon U.D., the district of the isolation of 1952.

One of the catering staff at hospital A continued to excrete the organism for a long time. Once amongst numerous attempts it was isolated from her husband. It was also isolated from the overflow from the septic tank at her home in September 1966.

In July 1967 the illness of the corridor maid and her husband during the time of the episode on ward 10 followed a meal of cold chicken cooked the day before and stored over a warm night in the larder of their home. Both of these fit adults were severely ill and it is probable that they had a large dose of organisms from the chicken; this maid had previously excreted *S. senftenberg* on two occasions in May and June 1965 but was not ill. Her friend the maid of ward 10 was also seriously ill at the same time with *S. senftenberg* in the stool, 7 days later a positive stool was obtained from her daughter.

Thus the *S. senftenberg* in the husbands of two of the hospital employees, the daughter of one and the overflow from the septic tank was related to the incidents in hospital A.

Not only were there only seven isolations of *S. senftenberg* from sources other than hospital A from December 1963 to March 1967 but the organism, if brought home by patients or staff, did not usually spread. One incident was noteworthy. The mother of one of the kitchen staff at A worked in the kitchen of hospital D. She and her family were examined repeatedly, but though her daughter continued to excrete *S. senftenberg* it was never isolated from the rest of the family.

DISCUSSION

There was little evidence of spread from person to person except in the hospital wards. After the hospital and kitchen had been closed, a new cleaner in the kitchen was found to be excreting *S. senftenberg* a week after arrival, which indicated a continuing source of infection in the kitchen.

One of us (P.B.C.), responsible for the hygiene of the kitchen, was persuaded during the survey of the kitchen in 1966 that lapses had been tolerated. Utensils, particularly meat knives, were old with split handles. The surface of the mobile table used for cutting meat and poultry was constructed of wood with deep fissures. Sponges were found on wash-hand basins and kitchen sinks for cleaning purposes. A can opener and slicing machine showed signs of hurried attention when last used. Cold-storage space was limited. A significant factor was the expanse of window facing the south-west, about 200 yards from a farmyard where feeding-meals for animals were compounded in an open shed. *S. senftenberg* is commonly found in various feeding-meals and much dust emanates from the machine during the process of grinding up grains and of compounding mixes. The kitchen of the hospital is converted from a ward hut and holes in the roof indicated where lights had been removed. However, samples of dust from the roof space and various other sites were never found to contain *S. senftenberg*.

The other factor considered as a source of the infecting agent was the poultry supplied to the hospital. The supplier obtained birds from two farms where there had been infection due to *S. senftenberg* in poultry (Hobbs & Hugh-Jones, 1969) *S. senftenberg* was isolated from litter sampled on two occasions from a turkey brooder house on a third farm 13 and 14 months after outbreaks of salmonella infection due to *S. senftenberg* had occurred.

The same supply of birds was used for two other hospitals in the group without ill effect. Nevertheless careful inquiry about the method of cooking turkeys—for example, in the affected hospital—indicated that rather short times at moderate temperatures were used in the ovens. In some instances incidents appeared to follow the introduction and preparation of turkey.

When the excretion of *S. senftenberg* was at its height, many symptomless excreters were found in the kitchen. It is probable that the food handlers and cleaners were infected from sources in the kitchen. The infection might have come from trolleys and waste foods passing from the wards to the kitchen for cleaning;

but since none of the returned food is likely to have been eaten by the kitchen staff such routes of spread would have given only very small doses of the organism. *S. senftenberg* is unlikely to cause infection in small doses, as shown by its absence in the general population, including the families of patients, and in other hospitals. It is more likely that there was a build-up of infection in the environment of the kitchen and in the foods going out from the kitchen in May and June 1964.

The reappearance of *S. senftenberg* from time to time in excreters was a puzzling feature. The differences between intermittent excreters and reinfection was difficult to assess. Those with positive stools were banned from the kitchen until six consecutive negative samples were obtained.

The invasion of this hospital by *S. senftenberg*, an organism of apparently low virulence, was most unusual. Although there were few deaths associated with it, nevertheless it caused much inconvenience to both hospital staff and patients. In particular, the training of nursing staff was interrupted and some careers disrupted.

It is suggested that the source of salmonellosis in hospitals should not only be sought for in the human population of the hospital but also in the bulk purchases of meat, poultry and other foodstuffs. Methods of preparation and the thoroughness of cleaning procedures may decide the fate of the organisms in the kitchen environment.

SUMMARY

Salmonella senftenberg was isolated from 168 patients and staff of a general hospital whereas it was isolated from only seven other sources (four of which were unquestionably associated with the hospital) in the surrounding area during the same period. It was isolated in the hospital in 19 of the 48 months of the period. Two clear-cut episodes were recognized against a background of sporadic isolation. Four-fifths of the patients and staff excreting the organism had no symptoms. Only two are known to have been severely ill, though in some patients very ill for other reasons life may have been shortened a little.

The original source of the organism, whether from the farm or from raw materials such as meat or poultry, was not found.

REFERENCE

HOBBS, B. C. & HUGH-JONES, M. E. (1969). Epidemiological studies on *Salmonella senftenberg*. I. Relation between animal foodstuff, animal and human isolations. *J. Hyg., Camb.* **67**, 81.

J. Hyg., Camb. (1969), **67**, 81
Printed in Great Britain

Epidemiological studies on *Salmonella senftenberg*

I. Relations between animal foodstuff, animal and human isolations

By BETTY C. HOBBS
Food Hygiene Laboratory, Central Public Health Laboratory, Colindale, London, N.W.9

AND M. E. HUGH-JONES
Department of Pathology and Epidemiology, Central Veterinary Laboratory, New Haw, Surrey

(*Received* 28 *June* 1968)

INTRODUCTION

Of all the *Salmonella* serotypes, *Salmonella senftenberg* is the one most commonly isolated in animal foodstuffs, but it constitutes less than 1 % of human or animal isolations (Taylor *et al.* 1965). This is true not only of the United Kingdom but of all other countries (van Oye, 1964). Even when isolated, it has seldom been associated with disease. It was therefore of some interest when a series of isolations of *S. senftenberg* started in 1963 at Ryhope Hospital, Sunderland, and developed into an epidemic of salmonellosis in April 1965 (Sanford *et al.* 1969).

BACKGROUND

A comparison of the weekly Public Health Laboratory Service and monthly Veterinary Investigation Service reports and the Ryhope Hospital records of salmonella isolations (1963–June 1967) show that the Ryhope outbreak was part of a widespread increase in *S. senftenberg* isolations (Fig. 1), but with the exception of outbreaks at Oxford and Sunderland the human isolations were of a sporadic nature. The Oxford outbreak occurred in November 1963, when *S. senftenberg* was isolated from a nurse, four children and an adult patient in the same hospital.

Thirteen animal strains were isolated in the period August 1964 to November 1965, and latterly three more were isolated in early 1967. Of the thirteen strains, six were from turkey poults on two farms, three from chicks, and the rest from a duck farm, a ewe flock and a dairy herd (Table 1). The isolations from foodstuffs only (Table 2) have been interpreted as 'isolation weeks'; that is, the number of weeks when a laboratory isolated the serotype in a product, as it was not possible from the reports to eliminate the possibility of multiple sampling and reporting from single shipments or plants; it is therefore an underestimate of the actual situation. The serotype was most frequently reported by the Preston, Colindale, Hull and Cardiff Public Health Laboratories in meat and bone meals in conjunction and separately. The individual isolations from sewers, abattoirs and rivers reflect both the animal and foodstuff conditions.

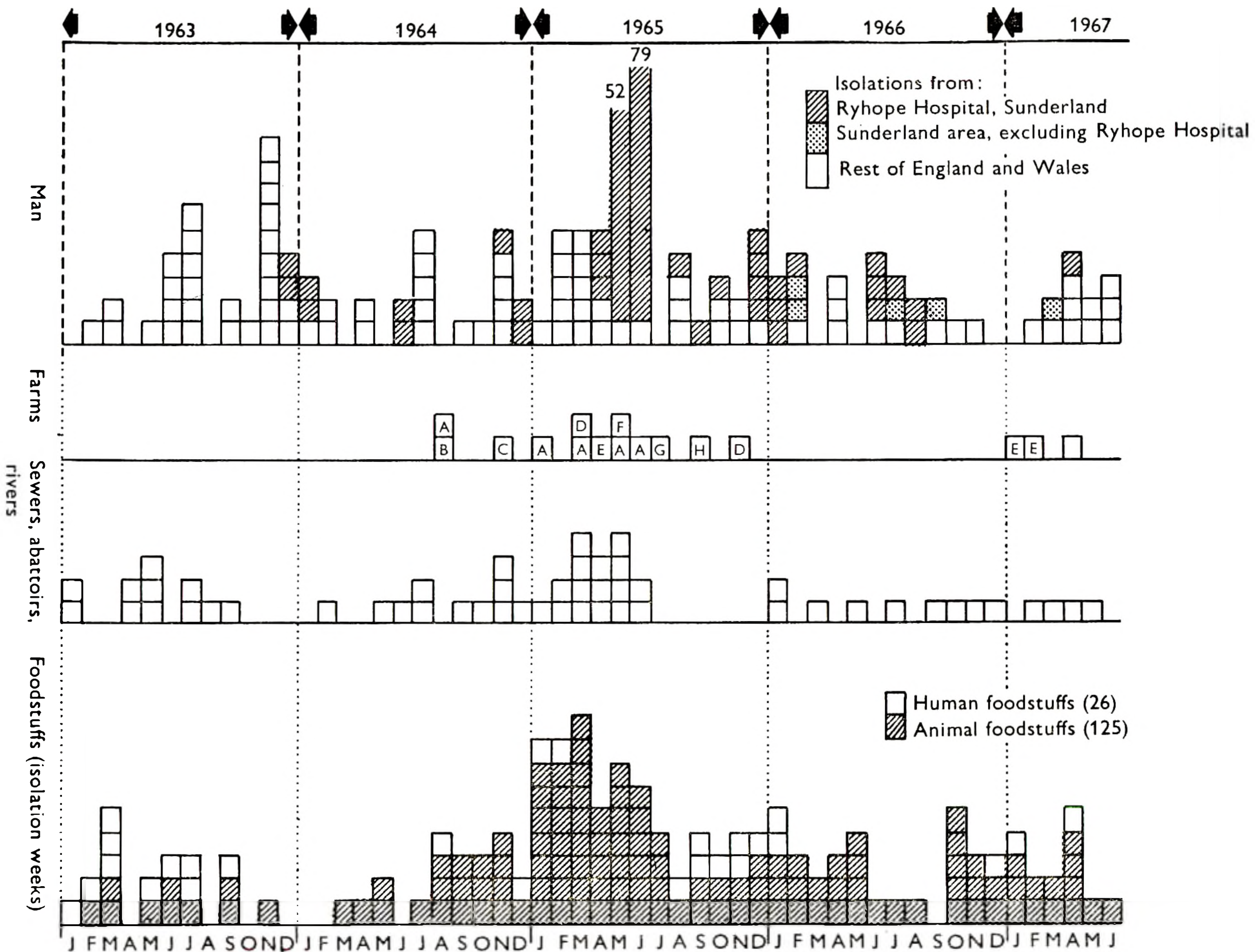


Fig. 1. Isolations of *S. senftenberg*, 1963 to June, 1967, as reported by the Public Health Laboratory Service, the Ryhope Hospital Laboratory and the Veterinary Investigation Service.

Table 1. *Isolations of* Salmonella senftenberg *from animals by the Veterinary Research and Investigation Services, Public Health Laboratory Service, and the Houghton Poultry Research Station, January* 1963 *to December* 1966

Month	Animals	Age	Supplier	Farm
		1964		
Aug.	Turkey poults	14 days	'Z' Hants.	Farm A (Yorks.)
	Broiler chicks	14 days	'Y' Mont.	Farm B (Ches.)
Nov.	Broiler chicks	5 days	Co. I., Yorks.	Farm C (Durh.)
		1965		
Jan.	Turkey poults	5 days	'X' Yorks.	Farm A
Mar.	Turkey poults	15 days	'Z' Hants.	Farm A
	Duck	?8 weeks	Farm D	Farm D, (Lincs.)
Apr.	Turkey poults	4–5 weeks	Farm E	Farm E, (Scot.)
May	Cows	Adult	.	Farm F, (Sussex)
	Turkey poults	13 days	'X' Yorks.	Farm A
June	Turkey poults	?	'X' Yorks.	Farm A
July	Ewe flock	Adult	.	Farm G (Yorks.)
	(? broilers)		('V' Yorks.)	(farm G_2)
Sept.	Layer chicks	6 days	'U'	Farm H (Essex)
Nov.	Ducks and geese	Not available	Farm D	Farm D

Table 2. *Isolations by 'isolation weeks'* of* Salmonella senftenberg *from various animal and human foodstuffs by Public Health Laboratories, January* 1963 *to June* 1967

(The figures are taken from the P.H.L.S. Weekly Salmonella Reports.)

Public Health Laboratory	No. isolation weeks	Feedstuffs	No. isolation weeks
Hull	25	Animal	125
Cardiff	25	Meat and bone meal	42
Colindale	24	Bone meal	26
Preston	19	Imported bone meal	10
Liverpool	13	Meat meal	17
Bristol	10	'Animal foodstuffs'	11
Ipswich	7	Fish meal	5
Northallerton	5	White fish meal	2
Glasgow	5	Imported fish meal	2
Conway	4	Protein concentrate meal	4
Newcastle	2	Cotton cake meal	2
Wakefield	2	Calf milk powder	1
Coventry	2	Feather meal	1
Manchester	1	Pig feed	1
Lincoln	1	Soya bean meal	1
Bournemouth	1		
Guildford	1	Human	26
Brighton	1	Imported Meat	11
County Hall	1	Dried Egg	8
Epsom	1	Coconut	5
Sunderland	1	Prawns, Yeast	2
Total	151	Total	151

* Number of weeks in which a laboratory isolated *S. senftenberg* in a product.

POSSIBLE SOURCES OF INFECTION

There were three questions to be answered in relation to possible sources outside Ryhope Hospital: was there a relationship between the known farm isolations and Ryhope Hospital, how did the serotype reach the farms, and what were the circumstances of its isolation on the farms? The last question will be covered in a separate paper (Hugh-Jones, 1969). Visits were made to all farms concerned, where possible, either by M.H.-J. or by the Veterinary Field Service. The relevant background information was provided by the laboratories and the Medical Officer of Health concerned. The feed companies were most helpful in providing details of feed constituents and the results of their own bacteriological examinations.

Relationship between known farm outbreaks and Ryhope Hospital

The hospital was provided with poultry by a firm which retails frozen dressed turkeys and slaughters chickens. The majority of the chickens were older laying birds which had been cleared from local battery units as they came off lay. The firm also bought in prepared chickens from a large concern which bred, reared, killed and packed their own birds. The turkeys came from three companies. There was an indirect connexion between one of these companies, company I, and farm C (as in Table 1). The turkeys hatched and reared by company I poultry hatcheries at their central establishment, and the broiler chickens at farm C, were fed on feeds from the company I feed compounders. The second of the three turkey companies regularly bought turkeys from farm E. There was a relationship between the turkeys on two farms on which *S. senftenberg* had been isolated and Ryhope Hospital.

On tracing the birds in the opposite direction—that is, from the farms—it became clear that although contracts controlled a fraction of the birds passing from farms to particular retail outlets, a significant proportion of birds could not be traced once they left the farm. This was not due to lack of co-operation by those concerned. These birds went either to large markets, such as Smithfield, or to wholesale chains which had widespread and numerous outlets. In either case an individual bird could be eaten anywhere in England.

Sources of infection on farms

On the eight farms recognized in 1964 and 1965 to have *S. senftenberg*, the animal species involved were turkey poults and growers, broiler and layer chicks, ducks and geese, ewes and cows. In spite of the variety of animals and ages suggesting a food-borne infection the possibility of a hatchery-disseminated infection was investigated. The true origins of day-old poultry are never completely certain because of hatcheries selling other birds to make up orders, but no poultry farm had knowingly received chicks or poults from hatcheries supplying any of the other farms (Table 1). The Veterinary Investigation Service salmonella records from January 1964 to June 1966, inclusive, were searched for other serotypes isolated from the relevant hatcheries and farms. As some of the flocks are accredited with

the Poultry Health Scheme, inquiries were also made from their records with the Divisional Veterinary Officers in their areas. Farms A, C and H had no histories of serotypes other than *S. senftenberg* being isolated. Farm B had an isolation of *S. typhimurium* in March 1965, from turkey growers. This agreed with the information gained directly from the farms when they were visited. But in January 1965, on farm A, 147 of 2600 poults had died by the fifth day. The initial diagnosis was 'failing to start to eat', but *S. senftenberg* was isolated from their yolk sacs. Inquiries showed that it was probable that the hatchery supplying the day-old poults had obtained infected eggs from farm E. Farms D and E are parts of large complexes with their own hatcheries and extensive trade in young stock. The duck farm D had a severe salmonella problem involving a number of serotypes but *S. senftenberg* was isolated once on the farm and from a duck in the local slaughter-

Table 3. *Constituents of poultry feeds*

+ = constant, ± = alternates, (+) = not constant. Under 'Scandinavian herring meal', N = ex Norway, I = ex Iceland, D = ex Denmark, S = ex Scotland.

	Maize/maize gluten	Sorghum	Soya	Sunflower/sesame	Wheat/oats/barley	Brewer's yeast	Distiller's grains	Vegetable fats	Fish meal (imported)	Scand. herring meal	White fish meal (Co. V)	White fish meal (Iceland)	Feather meal	Meat/meat and bone meals	Farm and feed at time of isolation(s)
Company I															
Broiler starter crumbs	+	+	+	.	.	.	+	+	.	NDI	+	+	.	.	C
Turkey prestarter	+	.	+	.	.	+	.	+	.	+	+	+	.	.	.
Turkey starter	+	.	+	.	.	+	.	+	.	+	+	+	.	.	.
Turkey grower 1	+	.	+	.	+	+	.	+	.	.	+	.	.	.	.
Turkey grower 2	+	±	+	.	±	+	.	+	.	.	+	.	.	.	.
Turkey finisher	+	±	+	.	±	+	.	+	.	.	+	.	.	.	.
Company II															
Broiler chick crumbs	+	+	.	.	+	.	.	+	.	NIS	+	.	.	.	G_2
Broiler finisher pellets	+	+	.	.	+	.	.	+	.	NIS	+	.	.	.	G_2
Company III															
Layer chick mash	+	+	+	+	+	.	.	+	±	N	.	.	.	.	H
Layer chick 1	+	+	+	+	+	.	.	+	±	±	.	.	.	.	.
Layer chick 2	+	+	+	+	+	.	.	.	±	±	.	.	.	.	.
Layer rearer	+	+	+	+	+	.	.	+	±	±	.	.	.	(+)	.
Broiler starter crumbs	+	+	+	+	+	.	+	+	±	N	(+)	.	.	(+)	B
Broiler pellets	+	+	+	+	+	.	.	+	±	±	.	.	.	(+)	.
Company IV															
(*a*) Scotland															
Turkey starter	+	+	+	.	+	+	+	.	.	+	.	.	.	.	E
Growing mash	+	+	+	.	+	.	.	.	.	+	.	.	+	+	E
(*b*) Midlands															
Turkey starter	+	.	+	+	+	.	+	.	.	+	.	.	.	.	A

house. Apart from the one time when an egg-transmitted infection may have occurred, there was no evidence to suggest that the infections were hatchery-disseminated. For example, *S. senftenberg* was isolated only from day-old poults subsequently delivered to farm A, while poultry from the same hatches delivered elsewhere were not infected.

With the exception of farm D, the names of the feeds being used at the time of the isolation of *S. senftenberg* were obtained from the farms. Direct inquiries were then made about the constituents of the chick and poult feeds as these would represent a known source, while the cows and ewes (farms F and G) might have been passing the serotype for some time before being identified as excretors. The companies supplying the feed at the times when outbreaks occurred in chicks and poults were questioned on the probable feed constituents at the time of the outbreaks (Table 3) and on *S. senftenberg* isolations from these constituents either in their own laboratories or others.

The different farm outbreaks had occurred between 1 and 2 years before these investigations were made and it was therefore fortunate that any useful information was still available. Farm C had sent twelve 5-day-old broiler chicks into a Veterinary Investigation Centre on 16 November 1964, and they had been fed on broiler starter crumbs made by company I. *S. senftenberg* was recovered from these chicks. The company I bacteriologist isolated *S. senftenberg* from a sample of company V white fish meal delivered to a southern subsidiary on 16 November. *S. braenderup* was also isolated. Deliveries made on 18 and 20 November contained *S. bredeney.* Another laboratory confirmed the 16 November findings but not the others. Three separate samples received by this laboratory about 20 December contained *S. cubana*. Further inquiries revealed that this particular fish-meal constituent contained only 10% of white fish meal, and that the protein level was made up with unspecified animal and vegetable proteins. All the turkey feeds made up by company I feed compounders contain company V white fish meal. It is of interest to note that the only poultry isolation of *S. braenderup* by the Veterinary Investigation Service in 1965 was in April from turkey growers on company I feed at a farm on contract to company I. The poulterer supplying Ryhope Hospital obtained turkeys from company I.

The broiler chicks on farm B were begun on company III broiler starter crumbs followed by broiler pellets. Gumboro disease broke out on the 14th day with a number of sudden deaths; *S. senftenberg* was also recovered from these birds. The starter crumbs contained both company V white fish meal and meat and bone meal, and the broiler pellets contained meat and bone meal. Unfortunately the company did not begin bacteriological examinations of their feed constituents until the autumn of 1965. The meat and bone meal could not be traced because there were various suppliers and there were delays between purchase, compounding and sale.

The birds from farm E were sent to a Veterinary Laboratory, it appears, as part of the routine examination of any dead or moribund birds, and *S. senftenberg* was isolated from them. The turkey poults were between 4 and 5 weeks old and had just been changed from a company IV turkey starter feed to a growing mash, which only differed from the former by containing feather meal and meat meal.

Unfortunately the Glasgow Public Health Laboratory only began to examine local animal feedstuffs in a regular manner in 1966 and the Scottish branch of company IV also only began to send them material at about the same time. J. E. Wilson (personal communication) exposed nineteen day-old turkey stags to an 18–24 hr. broth culture aerosol of *S. senftenberg* and recovered it from one of these birds 5 months later.

Farms A and H were provided with feeds containing Scandinavian herring meal which was common to all the farms. The chicks on farm H died in an outbreak of omphalitis (yolk-sac infection). The 14-day-old turkey poults from which *S. senftenberg* was initially isolated on farm A were part of a respiratory disease problem including aspergillosis. On this farm it was customary to move the poults at 5 weeks old to rearing sheds where they were put on a diet of company V white fish meal and grain.

The isolation of *S. senftenberg* on farm F from two random bovine dung samples occurred during a Medical Officer of Health's investigation of an outbreak of human dysentery. *Shigella sonnei* was recovered from two persons and *S. senftenberg* from six of thirteen persons examined. No disease was noted in the cows at the time nor was the source of infection discovered. The ewe flock on farm G was grazing in a field 3 weeks after it had been spread with manure from a broiler flock on a separate farm G_2. No disease was reported in these birds and 5363 broilers were reared from 5610 day-old chicks. Their diet had contained both Scandinavian herring meal and company V white fish meal.

There is thus circumstantial evidence linking animal feedstuffs with the farm outbreaks; white fish meal, known to have been contaminated and readily available could have been responsible. Of the five turkey and chicken outbreaks, all the birds received Scandinavian herring meal, and Norwegian herring meal in three outbreaks. Two turkey farms, C and E, could be linked to Ryhope Hospital, but because of the wide and increased prevalence of contaminated animal foodstuffs other sources of infection cannot be excluded.

DISCUSSION

S. senftenberg is widely disseminated through the country in animal foodstuffs and especially meat and bone meals. The records from the farms involved in the August 1964 to November 1965 incident would suggest that outbreaks in animals on farms can be used as indicators of the increased incidence of a serotype, although clinical salmonellosis was not associated with the isolations of *S. senftenberg*. Overt and covert individual outbreaks would increase the number of animals excreting the serotype and the duration of excretion. A general rise in prevalence of *S. senftenberg* in abattoirs and foodstuffs can be reflected in the general human population (e.g. in 1963 and 1964–65), but without the acute outbreak at Ryhope Hospital there would have been only a slightly raised incidence in the human population. Thus there is a potentially hazardous situation when a serotype is more generally available. As this can occur with *S. senftenberg*, which is relatively rare in animal and human populations, owing possibly to a lack of invasiveness or of adaptation

or to some other cause, better adapted serotypes might be expected to behave in a similar way but with more serious consequences.

SUMMARY

It was possible to demonstrate from retrospective records that a link existed between contaminated animal feedstuffs, turkeys and an outbreak of *Salmonella senftenberg* infection at Ryhope Hospital. Possibly infected or contaminated turkeys were supplied to the hospital from two turkey farms in one of which the birds were fed on contaminated white fish meal and the other had had infected birds.

This work was carried out months after the events described and would not have been possible without the help and co-operation of the Public Health Laboratory Service, The Veterinary Field, Investigation and Research Services, Houghton Poultry Research Station, The Medical Officer of Health for Brighton, Dr Rosetta Parker, and the various feed companies involved, who very kindly made their records available to us.

REFERENCES

HUGH-JONES, M. E. (1969). Epidemiological studies on *Salmonella senftenberg*. II. Infections in farm animals. *J. Hyg., Camb.* **67**, 89.

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

SANFORD, D. A., LESLIE, D. A., MCKEON, J. A., CRONE, P. B. & HOBBS, B. C. (1969). *Salmonella senftenberg* in the Sunderland area. *J. Hyg., Camb.* **67**, 75.

TAYLOR, J., LAPAGE, S. P., BROOKES, M., KING, G. J. G., PAYNE, D. J. H., SANDIFORD, B. R. & STEVENSON, J. S. (1965). Sources of salmonellae. *Mon. Bull. Minist. Hlth* **24**, 164–229, 237–77.

J. Hyg., Camb. (1969), **67**, 89
Printed in Great Britain

Epidemiological studies on *Salmonella senftenberg*

II. Infections in farm animals

By M. E. HUGH-JONES
Department of Pathology and Epidemiology, Central Veterinary Laboratory, New Haw, Surrey

(*Received* 28 *June* 1968)

INTRODUCTION

According to Taylor (1967) and van Oye (1964) the most frequently isolated salmonella serotypes in any one country are characteristic of that country and not liable to extreme changes over short periods of time, although the order will change and some serotypes will disappear and appear in time. Amongst these are the serotypes most capable of animal-to-animal spread, such as *Salmonella typhimurium*, *S. dublin* and *S. choleraesuis*. The less commonly isolated serotypes are more necessarily involved in food cycles with sporadic outbreaks the frequency of which appears to depend on the current level of contamination of animal feed and human foodstuffs. These seldom cause disease on farms (Gibson, 1965) and differ experimentally in their ability to do so (Vestal & Stephens, 1966). This also appears to be true for human disease; Szanton (1957) and Watt *et al.* (1958) have reported infection in infants with *S. oranienberg* and *S. tennessee* without clinical disease, and McCall *et al.* (1966) have reported clinical symptoms to be minimal or absent with other serotypes. The production of clinical symptoms (recognizable 'salmonellosis') is dependent not only on certain characteristics such as age of the individual and dosage but also on intercurrent infection, as seen in hospital outbreaks (Tin Han, Sokal & Neter, 1967, Datta & Pridie, 1960), farm outbreaks (Gibson 1965) or experimental malaria (Kaye, Merselis & Hook, 1965) or coccidiosis (Stephens & Vestal, 1966). Adverse environmental conditions such as cold or lack of feed and water have been shown to affect the clinical disease rate of animals by Previte & Berry (1962) and Bierer & Eleazer (1965), but this was denied by Morrello, Digenio & Baker (1965). The reduction of intercurrent infection, by the use of coccidiostats and tylosin, appears to reduce the attack rate in experimental and field conditions (Bierer, 1961; Ridgway & Ryden, 1966).

Since *S. senftenberg* is so widespread in animal feedstuffs and yet so infrequently isolated from men and animals, it is of some interest to know why it should be isolated at all. While tracing the sources of the animal infections in a series of *S. senftenberg* isolations, reasonably complete histories were obtained from six of the eight farms by visits to the farms and examination of their records maintained by the Veterinary Investigation Service and Veterinary Field Service. Salmonellosis in poultry up to 14 days old is characterized by failure to thrive, inappetence, diarrhoea, and increasing numbers of deaths; after death the carcass has a septicaemic appearance, enlarged congested liver with focal necrosis, some myocarditis and caecal casts.

FARM INVESTIGATIONS

Farms B–H

The disease histories and details of animal feeding on these farms are given in a previous paper (Hobbs & Hugh-Jones, 1969) and the possibility of a feed contamination discussed. In the farms from which *S. senftenberg* had been isolated, no disease was reported from a dairy herd, there was a chronic diarrhoea of unknown cause in a ewe flock, and Gumboro disease, starvation syndrome and omphalitis in each of three poultry flocks. In these instances *S. senftenberg* did not appear to have acted as a primary pathogen, if as a pathogen at all.

Farm A

We are fortunate to collect a complete series of post-mortem examination and veterinary field service reports from this farm covering the period from March 1963 to August 1966. All the post-mortem examinations had been done by one laboratory worker.

This farm is the headquarters of a five-farm complex, each farm with a commercial turkey flock; farm A also has a breeding flock in the Poultry Health Scheme, separate from the commercial flock, producing fertile eggs for a hatchery. The commercial farm A flock is about a quarter of a mile from the main buildings and has a separate staff. Originally the farm produced forced spring rhubarb but the market for this product fell and the field buildings were used for raising turkeys. Infection with *S. senftenberg* was limited to the commercial flock at farm A and was not found at any of the other four farms. The birds arrive as day-old poults and are placed in a pair of central brooder houses with a common feed store. The houses are in moderate repair with wooden walls and compacted earth floors; woodshavings are used for litter; both piped and bottled water is available. The roof of the feed stores appeared to be sound. The poults receive company IV 'turkey starter' (Hobbs & Hugh-Jones, 1969) for the first 5 weeks in self-feeders which are supposed to be checked and the faeces removed every day. At 5 weeks old the birds are moved into growing or fattening sheds, and fed on a growing ration of company V white fish meal and grain (soya, maize, barley or oats, vitamin and minerals). They are slaughtered in these sheds when 18–20 weeks old after 24 hr. starvation, but birds from certain hatcheries are returned alive in crates for slaughter elsewhere. Slaughtering starts in mid-April and is continuous until mid-December, when left-over birds are sold locally. Empty houses are cleaned out, sprayed with 'Hydrox' mixed with diesel oil and left empty for no less than 1 week. All the houses are empty over Christmas and the brooder houses are re-stocked in January.

It is only in hindsight that it could be suggested that the presence of *S. senftenberg* contributed to the ill-health of turkey poults on farm A. Frequently the post-mortem picture was of a non-specific nature, typical of 'chilling' and 'not starting'. During 1963 aspergillosis was diagnosed twice in growers and poults. In April 1964 the flock was tested serologically for *S. pullorum* with negative results. *S. senftenberg* was isolated for the first time in August 1964. The initial deaths had

been normal among the 2000 day-old poults but rapidly increased from the tenth to the fourteenth day. Furazolidone treatment was started, the deaths tapered off with a 6 % mortality and the birds reared satisfactorily. Aspergillosis was diagnosed on the basis of mycotic abscesses in the lungs and air sacs and *S. senftenberg* was isolated from the swollen livers of all the four dead birds examined.

In January 1965, 2600 day-old poults arrived and by the fifth day 147 birds had died. Post-mortem examination showed that they had not been eating; the lungs and kidneys were congested; the gall bladders distended; the yolk sacs were being absorbed and, besides *Escherichia coli*, *S. senftenberg* was isolated from the yolk sacs. The initial diagnosis was 'delay in starting to eat'. The hatchery was visited by the Veterinary Field Service and found to have 'the highest standards of hygiene and organization'. However, the hatch providing these birds was made up of 2600 eggs from farm E in Scotland and 1484 of the hatchery's own eggs (*S. senftenberg* was isolated from farm E for the first time in April 1965). These eggs were incubated in separate machines and 49 % and 68 % hatched respectively. Another farm received 480 poults from the second day's hatch and suffered an over-all 43 % mortality with forty-six dying in the first 4 days. Unfortunately none of these birds were available for bacteriological examination. It seemed probable that the hatchery might have obtained infected eggs from farm E, but other factors could have been involved.

Late in February 1965, 1350 day-old poults were mixed with 1650 week-old poults from the same hatchery and the birds began to die, although on a furazolidone-supplemented diet. Of the 10 week-old birds examined, one had an incorrectly absorbed yolk sac, and the others congested lungs and kidneys, distended gall bladders and empty gizzards; such lesions are associated with birds 'not starting'. *E. coli* was recovered from the lungs and kidney, but the intestines were not examined bacteriologically. This flock was seen again when 5 weeks old and proved to be unthrifty and unevenly grown. Seven were examined out of the forty-one that had died; the carcasses were rather wet and congested and the gizzards were a little enlarged; five had air-sacculitis, thin-walled intestines, and enlarged and congested kidneys. *S. senftenberg* was isolated from the intestines. It was noted at the time that the respiratory lesions were insufficient to have caused death. The hatchery was visited again but no problems had been reported with the poults from the same hatch on other growing farms.

In March a delivery of 5600 day-old poults from a different hatchery arrived; 3600 went to another farm and remained healthy, while 2000 stayed on farm A. They were cared for by the same poultry man as the February birds and were in the same group of buildings. The poults were severely debeaked on day 12 and 3 days later 287 birds died (see Fig. 1), although furazolidone was added to their food from day 13. The poultryman reported that the birds were found dead with no apparent symptoms. Of the ten 15-day-old poults examined, three showed pale areas on the gizzard muscles, one had small 'brownish' kidneys, one only had food in its crop and their intestines were flaccid; four had swollen livers and inflamed kidneys, and the rest congested kidneys only. *S. senftenberg* was isolated from all three livers examined. When seen by a Field Officer on day 23 there were no ailing

birds in the flocks. It seemed that the severe debeaking of the flock with resultant tissue damage, shock and depriving the poults of food and water, caused an abnormally large number of deaths with post-mortem lesions similar to those of salmonellosis.

In May 3000 day-old poults arrived from the same hatchery as the January and February birds and they were immediately started on a furazolidone-supplemented diet. Deaths occurred as shown in Fig. 1 and at 1 month old the flock was seen to be unthrifty, with birds of different sizes, but with no symptoms noticed by the poultryman other than sudden deaths. A post-mortem examination of birds

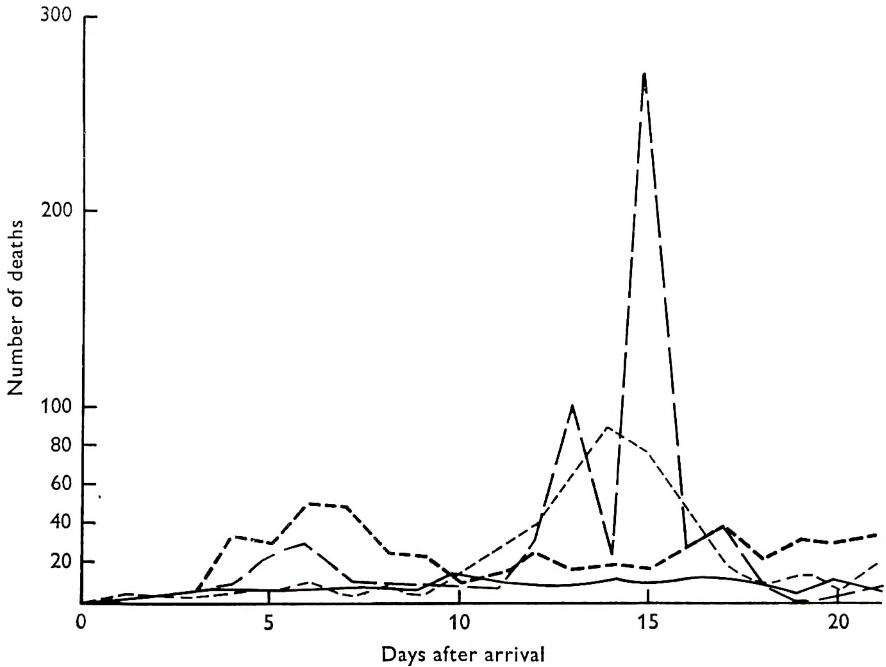


Fig. 1. Deaths in batches of day-old poults at farm A.
—— —— 2000 which arrived 1 March 1965; mortality 30·8 %.
– – – – – 3000 which arrived 10 May 1965; mortality 16·2 %.
- - - - - 2000 which arrived 7 July 1965; mortality 22·5 %.
———— 2828 which arrived 21 July 1965; mortality 5·5 %.

13 days old showed them to have a mild enteritis, diarrhoea, enlarged gall-bladders and congested livers; in birds dying at 21 days there was also congestion of the lung and kidney and diarrhoea; one of the eight birds examined had a curled tongue. *S. senftenberg* was isolated from the intestines of the 13-day-old carcasses but not from those of the 21-day-old birds. The hatchery was visited again and it was observed also that birds from the same hatch sent to other farms had remained healthy. A year later, when the farm was visited, the commercial flock showed chronic respiratory symptoms, and considering the earlier history of this flock, it may be assumed that there had been a constant respiratory problem on this farm.

In June 1965 *S. senftenberg* was isolated again from week-old poults that had 'failed to start', from which time the manager ceased to send carcasses for examina-

tion, although deaths continued to occur. However, in late July the normal number of deaths occurred in a new batch of poults. When the brooder house was re-stocked after the 1965 Christmas holiday, normal death-rates occurred among the birds, and *S. senftenberg* was not recovered from any dead birds. The known isolations of *S. senftenberg* occurred from August 1964 to June 1965 from birds in both brooder houses, and in August and September 1966 *S. senftenberg* was recovered from the earth floors under the existing litter by the Food Hygiene Laboratory. Unfortunately the source of infection on this farm was not discovered.

DISCUSSION

All these isolations of *S. senftenberg* suggest that it is usually found by chance as a result of the bacteriological examination of animals with other disease conditions. *S. senftenberg* is an accidental finding. But under circumstances of extreme 'stress', such as debeaking very young turkey poults, it may contribute to the subsequent mortality if not actually produce salmonellosis. It may also aid other secondary pathogens or disease conditions, such as a chronic low-level respiratory problem, or cold brooder conditions, in producing a non-specific but significant number of deaths in poults, but of itself not be pathogenic enough to give such deaths the characteristics of a 'disease'.

SUMMARY

Between August 1964 and November 1965 *Salmonella senftenberg* was isolated from poultry, sheep and cattle on eight farms in England and Scotland. From an analysis of the case records its presence would appear to be incidental; but it may contribute to poultry mortality by acting in conjunction with other intercurrent infections or following stressful events such as severe debeaking or cold brooder conditions.

I should like to thank the officers of the veterinary field investigation and research services who made available their records and notes for this work, and who visited the farms and hatcheries, but especially Mr R. Duff, Veterinary Laboratory, Lasswade, for his reports on the turkey poults from farm A; also Dr Betty Hobbs and Mr B. Hanson for their valuable criticisms.

REFERENCES

Bierer, B. W. (1961). Nitrofuran coccidiostat aids in control of pullorum disease under stress conditions. *J. Am. vet. med. Ass.* **139**, 238–9.

Bierer, B. W. & Eleazer, T. H. (1965). Clinical salmonellosis accidentally induced by feed and water deprivation of one-week-old broiler chicks. *Poult. Sci.* **44**, 1606–7.

Datta, N. & Pridie, R. B. (1960). An outbreak of infection with *Salmonella typhimurium* in a general hospital. *J. Hyg., Camb.* **58**, 229–41.

Gibson, E. A. (1965). Reviews of the progress of dairy science. Section E. Diseases of dairy cattle. Salmonella infection in cattle. *J. Dairy Res.* **32**, 97–134.

Hobbs, B. C. & Hugh-Jones, M. E. (1969). Epidemiological studies on *Salmonella senftenberg*. I. Relation between animal foodstuff, animal and human isolations. *J. Hyg., Camb.* **67**, 81.

Kaye, D., Merselis, J. G. & Hook, E. W. (1965). Influence of *Plasmodium berghei* infection on susceptibility to salmonella infection. *Proc. Soc. exp. Biol. Med.* **120**, 810–13.

MCCALL, C. E., COLLINS, R. N., JONES, B. D., KAUEMANN, A. F. & BRACHMAN, P. S. (1966). An interstate outbreak of salmonellosis traced to a contaminated food supplement. *Am. J. Epidem.* **84**, 32–9.

MORELLO, J. A., DIGENIO, T. A. & BAKER, E. E. (1965). Significance of salmonellae isolated from apparently healthy mice. *J. Bact.* **89**, 1460–4.

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

PREVITE, J. J. & BERRY, L. J. (1962). The effect of environmental temperature on the host–parasite relationship in mice. *J. infect. Dis.* **110**, 201–9.

RIDGWAY, F. & RYDEN, R. (1966). Resistance studies of *Salmonella typhimurium* in chicks on a tylosin feed. *J. comp. Path.* **76**, 23–30.

STEPHENS, J. F. & VESTAL, O. H. (1966). Effects of intestinal coccidiosis upon the course of *Salmonella typhimurium* infection in chicks. *Poult. Sci.* **45**, 446–50.

SZANTON, V. L. (1957). Epidemic salmonellosis. A 30-month study of 80 cases of *Salmonella oranienberg* infection. *Pediatrics, Springfield* **20**, 794–808.

TAYLOR, J. (1967). Salmonellosis. II. Public health aspects. *Vet. Rec.* **80**, 147–54.

TIN HAN, SOKAL, J. E. & NETER, E. (1967). Salmonellosis in disseminated malignant diseases. A seven-year review (1959–1965). *New Engl. J. Med.* **276**, 1045–52.

VESTAL, O. H. & STEPHENS, J. F. (1966). The relative pathogenicity of selected paratyphoids for chicks. *Avian Dis* **10**, 502–7.

WATT, J., WEGMAN, M. E., WHARTON BROWN, O., SCHLIESSMAN, D. J., MAUPIN, E. & HEMPHILL, E. C. (1958). Salmonellosis in a premature nursery unaccompanied by diarrheal disease. *Pediatrics, Springfield* **21**, 689–705.

J. Hyg., Camb. (1969), **67**, 95
Printed in Great Britain

Protection from microbial contamination in a room ventilated by a uni-directional air flow

O. M. LIDWELL AND A. G. TOWERS*
Cross-Infection Reference Laboratory, Colindale Avenue, London, N.W. 9

(*Received* 12 *July* 1968)

There are a number of medical situations in which the protection of a patient from infection is of particular importance. For example, patients receiving cytotoxic drugs, suffering from extensive burns or being nursed in intensive care units after serious operations or major injury are unusually likely both to become infected and to suffer from the more serious clinical consequences of infection. The absolute and relative importance of the many possible sources and routes of infection for these patients is largely unknown and certainly varies for different categories of patients and in different environments. Total isolation from all extraneous sources of micro-organisms has been attempted by enclosing the patient in a plastic tent, carrying out all manipulations through glove ports, and using double-ended hatches for passing in sterile materials and removing wastes. The extensive use of systems of this kind lead to substantial nursing difficulties and to psychological problems for some patients. (Levitan *et al.* 1968).

In so far as the infective agent is airborne and is either inhaled by the patient or settles on a sensitive area, the risks of infection can be reduced by ventilation. The traditional methods of ventilating a space produce or permit sufficient turbulent circulation of the air to distribute any contamination dispersed into the air relatively evenly throughout the room and subsequent elimination follows a logarithmic course, i.e. successive fractional reductions in the contamination level take equal times and absolute removal theoretically requires infinite time. Since sedimentation is unaffected by the ventilation process, increases in the amount of ventilation produce a less-than-proportionate increase in the rate of clearance. To take a simple example, if the dispersed particles have a sedimentation rate of 1 ft./min.—a common average value for naturally dispersed airborne particles carrying bacteria—the time for the initial numbers to fall to one-tenth will be 23 min. in an unventilated room 10 ft. high, 12 min. if ventilation is provided at 6 air changes/hr. and about 5 min. if this is increased to 20 air changes/hr. If, however, the ventilating air could be induced to move across the space in one direction only, without any turbulent mixing, complete removal would be attained in the time taken for the air to traverse the space once. Attempts to apply this principle to the ventilation of surgical operating rooms with the limited volumes of ventilating air considered practicable proved to be ineffective, since the translational velocities that could have been produced by the introduced air, between 2 and 5 ft./min., were smaller than the turbulent velocities produced by thermal

* Working on a Ministry of Health Research Grant.

effects and the movement of persons (Lidwell, Richards & Polakoff, 1967). If however, the ventilating air volumes can be increased by a factor of 10 or more the effect of these disturbances is largely eliminated.

Industrial requirements for dust-free spaces, stimulated by the demands of the U.S. space programme, have led to the development in recent years of directed-flow ventilation systems employing both vertical and horizontal air flow in the range 50–100 ft./min. These are often described as 'laminar flow' systems, but as they do not fulfil the aerodynamic conditions for genuine laminar flow it seems desirable to avoid using this term as far as possible. The turbulent air movements in such systems are, however, sufficiently small for practically complete removal of air-dispersed particulates to be obtained with a single air change. The disturbance of the air flow by objects introduced into the room does not significantly detract from their performance in this respect so long as the dimensions of these objects are small in relation to those of the room. By nursing patients in a room ventilated in this way it should be possible practically to eliminate the risk of airborne infection. In addition to the possible clinical benefit that this might bring, such an environment would greatly facilitate the study of other routes of infection. If the potentially ubiquitous airborne route could be eliminated, details of the paths of contact transfer in a nursing situation could be more easily evaluated. With a view to exploring these possibilities, the Ministry of Health in 1967 acquired a clean room, based on an industrial model, with horizontally directed air flow.

We report here the results of a preliminary investigation carried out in this room using artificially generated bacteria-carrying airborne particles and exploring the dispersal of these with different air-flow velocities and with various degrees of active movement in the room. We were able to introduce very large numbers of particles at any one point, usually about 30 million in a single experiment. It was then possible to detect as few as 1 in 10^7 of the dispersed particles reaching a square foot of horizontal surface exposed anywhere within the room.

METHODS

Description of the room and ventilating system

A diagram of the room in section is given in Fig. 1, together with a schematic representation of the ventilating and air conditioning plant. The floor, covered with vinyl sheet, was 16 ft. 8 in. long by 10 ft. wide. The room was 8 ft. 2 in. high and one end was entirely composed of a bank of filters through which the ventilating air entered at a uniform velocity over the whole wall area. This velocity could be adjusted up to 100 ft./min. by controlling the recirculating fans. The air was extracted from the opposite end through a series of louvres. These covered the entire wall surface and could be adjusted so that the outflow velocity was also uniform over the whole area. The two long walls were covered with Formica, except for the windows, and the ceiling, below the lights, was formed by double-walled panels covered with thin polyvinyl sheet. Tests with smoke generated from a stick dipped in titanium tetrachloride and with a vane anemometer showed that, at air velocities of 60 or 100 ft./min. air flow was nearly horizontal and at a con-

stant velocity throughout the room. At a velocity of 35 ft./min., however, when the refrigeration unit was in operation, uneven temperature distribution in the incoming air resulted in significant departures of the air flow lines from the horizontal. At 20–25 ft./min. these disturbances were much more marked and back flow could be detected in some regions. All tests done at these low velocities were therefore carried out with the refrigeration unit switched off. Refrigeration is normally necessary in a room of this type in order to remove the heat generated by the recirculating fans. The divergence of the smoke tracks showed that under all conditions small-scale turbulence was present, but the included angle of the visible smoke trail, averaged over some minutes, was not normally as much as 10°.

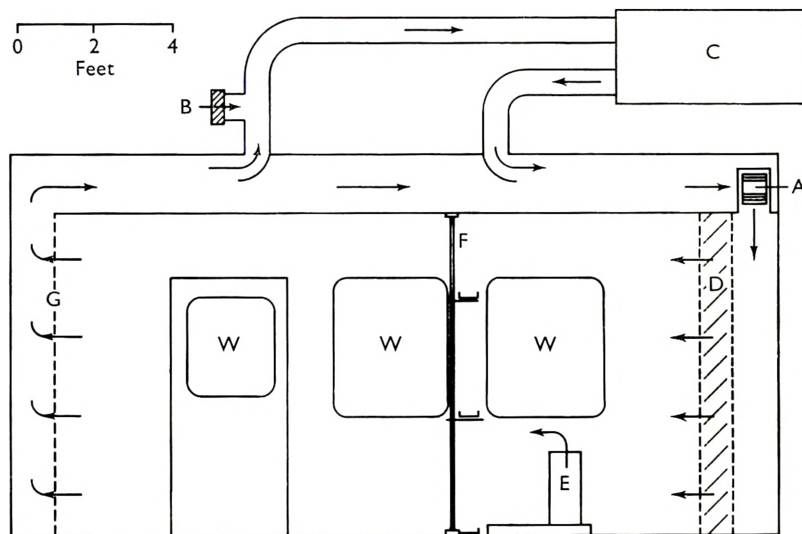


Fig. 1. Section of room. A, Recirculating fan; B, make-up air inlet; C, air-conditioning unit (temperature and humidity control, including a refrigeration unit); D, filter bank (prefilter followed by high efficiency filter); E, airborne particle generator; F, stand with sampling plates; G, return grille.

Production of airborne bacteria-carrying particles

In order to simplify calculations involving both the numbers of particles in a given volume of air and the numbers settling into a specified area, it is convenient to use particles of uniform size. Studies in a variety of environments have shown that a common value for the median diameter of bacteria-carrying particles in occupied environments is around 13 μ, assuming unit density for the particles, or a settling rate in air under normal ambient conditions of about 1 ft./min. (Noble, Lidwell & Kingston, 1963). The most satisfactory method of producing such particles is the air-driven spinning top (May, 1949). If this is operated at an air pressure of about 7 lb./sq.in., the primary droplet produced is of the order of 40 μ in diameter. When spraying a fluid such as nutrient broth with a solids content of some 2·5 %, the diameter of the resulting 'dried' particle will be rather over 10 μ. At 50 % relative humidity drying is not complete and the particles resulting from spraying a broth suspension of *Bacillus subtilis* spores had an average settling rate

in air of about 1·1 ft./min., or an estimated diameter, assuming that the particles were of unit density, of 13·5 μ.

To avoid uncertainties, due to the break-up of particles in a fluid medium, the density of the spore suspension was selected so as to give only a small probability that any sprayed particle would carry more than a single spore. The spore suspension used was prepared by growing *B. subtilis* var *niger* N.C.T.C. 10073, Detrick strain, according to the method of Beeby & Whitehouse (1965). The viable count of the stock suspension in distilled water was estimated by depositing with a calibrated pipette drops of suitable dilutions on nutrient agar plates. Characteristic pigmented colonies are formed after overnight incubation at 37° C. The suspension was stored in the refrigerator at 4° C. and diluted with nutrient broth

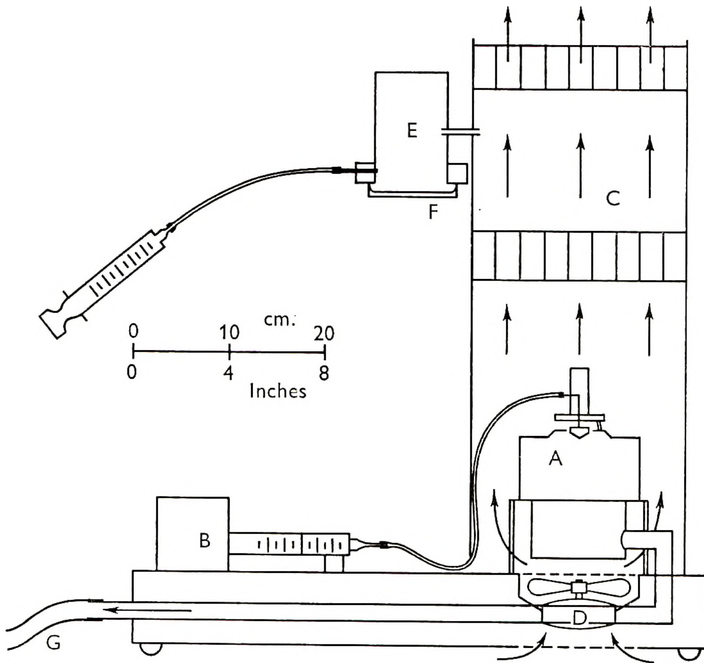


Fig. 2. Airborne particle generator. A, Spinning top (May, 1949); B, mechanically driven syringe for feeding suspension; C, vertical wind-tunnel with baffles to reduce turbulence; D, fan; E, sedimentation chamber for sampling dispersal cloud; F, sampling plate for cloud; G, tube to lead effluent air from spinning top, with satellite particles, out of room.

before each test-run to a final concentration of 6×10^6 colony-forming units per ml. Since a 40 μ diameter droplet has a volume of approximately 3×10^{-8} ml. the probability that any droplet will contain a viable spore is approximately 17 %, and less than 10 % of the infected droplets would be expected to carry more than one viable spore.

Dispersal of the bacteria-carrying particles

In order to introduce the particles into the room at a determined point they were generated at the base of a short vertical wind-tunnel. The diameter of this was made as small as possible consistent with avoiding impingement of the sprayed

droplets onto the walls, and air was introduced at the base at a sufficient rate to ensure an adequate air flow up the tunnel to carry the dried droplets with it and discharge them from the upper end of the tube. The arrangement used is shown in Fig. 2. The diameter of the tunnel was 9 in. (23 cm.) and air was blown in at 210 l./min. The compressed air supply to the rotor at 7 lb./sq.in. (0·46 kg./cm.^{2}) was equivalent to 30 l./min. free air and the induced efflux from the apparatus was 70 l./min. The net flow up the tunnel was therefore approximately 170 l./min., giving a discharge velocity of 7 cm./sec. or about 14 ft./min. At this velocity the discharge did not significantly affect the air-flow lines in the room, and the effects on these of the apparatus itself were small. The spore suspension was fed on to the centre of the spinning disc at a constant rate, 0·35 c.c./min., by a mechanically

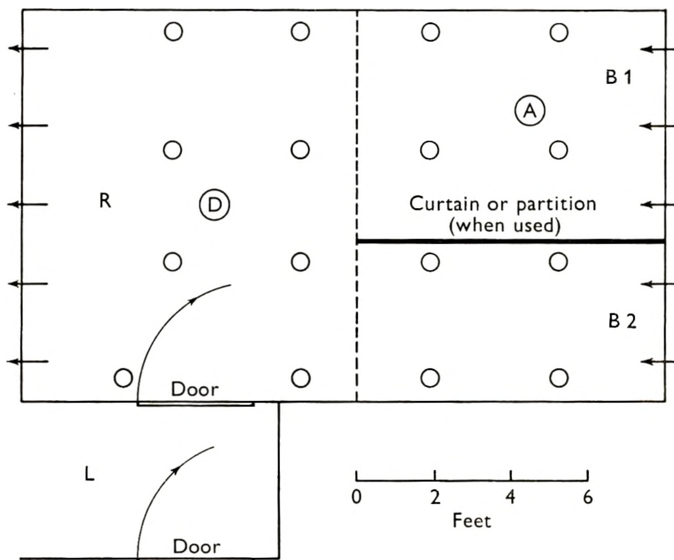


Fig. 3. Plan of room. B 1, B 2, Notional bed areas; R, rear area of room downwind of bed areas. A, dispersal point in B 1; D, dispersal point at rear of room. The unlabelled circles mark the position of the sixteen stands each carrying up to three 5½ in. diameter Petri dishes (F in Fig. 1).

driven syringe. As a check on the rate of dispersal of the spore particles, two 10 c.c. portions of the particle-laden air were withdrawn into the sedimentation chamber shown in the figure shortly after the start and shortly before the end of each period of spraying. The median number of colonies found on the settling plates from 35 tests was 186. This corresponds to a dispersal rate of $(186 \times 170 \times 10^3)/20 = 1{\cdot}6 \times 10^6$ spore-bearing particles per minute, compared with the delivery rate of $0{\cdot}35 \times 6 \times 10^6 = 2{\cdot}1 \times 10^6$ viable spores per minute.

Sampling method

The use of a homogeneous bacterial cloud leads to an exact equivalence between sampling by volumetric or settling methods whatever the age of the cloud. In these circumstances, the exposure of open plates containing culture medium allows

samples to be obtained simultaneously from many positions in the room without disturbing the air-flow pattern by introducing large amounts of apparatus.

Petri dishes, $5\frac{1}{2}$ in. in diameter, containing nutrient agar were exposed at sixteen points disposed in a uniform grid over the plan of the room (see Fig. 3). Three plates were exposed at each point, one 6 ft. above the floor, one 3 ft. above the floor and one on the floor itself. The upper two of each set of three plates were supported on small horizontal platforms made of aluminium sheet attached to $\frac{1}{2}$ in. diameter vertical rods. The plates were uncovered, starting from the upstream end of the room at the beginning of each experiment, and normally covered again in the reverse order 5 min. after the conclusion of the spraying period. In order to check the rate of contamination due to manipulation, a total of 133 plates were exposed and recovered at various times during the course of the experiments without any spraying of spores. In all, fifty-three colonies of pigmented subtilis were found on twenty-seven of the plates after incubation. The expected rate of accidental contamination during the experiments was therefore 0·40 colonies per exposed plate.

The exposed surface area of each plate was approximately $\frac{1}{6}$ sq.ft., so that the total sampling area was 8 sq.ft. or about one twentieth of the total floor area of the room.

Experimental procedure

The room was cleaned initially and between each group of two or three trials by swabbing the floor with detergent solution, the walls and plate-carriers were similarly treated after any major rearrangement of equipment within the room. The two people who entered the room wore clean laboratory coats and plastic overshoes but took no other special precautions against contamination. Trials were carried out at four air-speeds measured by a vane-type anemometer across the central transverse plane of the room. The speeds used were 100, 60, 35 and 22 ft./min., and variations in time and space across this plane did not exceed $\pm 20\%$.

In addition, comparative measurements were made by means of two systems that produced highly turbulent conditions of air movement in the room. The first was provided by a small fan unit placed in the centre of the room 7 ft. above the floor which recirculated the air through a high-efficiency filter at 130 cu.ft./min., equivalent to an air change rate of 6/hr. The recirculating fans were switched off when this system was in use, and the return grilles were blanked off with polythene sheet. The second was obtained from a portion of the recirculating plant: 1600 cu.ft. of air/min. were delivered into the room through a hardboard funnel arranged to produce a highly turbulent air-flow pattern in the room without excessive air velocities below the 6ft. 6 in. level. The return grilles were mostly blanked with polythene sheet, leaving two outflow apertures about 2×2 ft. each at the bottom corners. This rate of air flow corresponds to an air change rate of 75/hr.

As the rate of clearance of the air-borne spores was much slower with these systems than with the directed air flow, the period of spraying was reduced from the usual 20 min. to 1 or 2 min. only, and a substantial period (20 min. and

10 min. respectively) was allowed after the conclusion of spraying before the exposed plates were re-covered.

In addition to varying the ventilation arrangements, two positions were used for dispersal of the particles, and a curtain or a solid partition was used to divide up the interior of the room during some experiments. Figure 3 shows the position of these.

During the entire period of exposure of the plates, the two people in the room moved about according to one or other of four standardized patterns. These were: (1) moving about continuously in the rear half of the room only, (2) moving about continuously in this area and in the left-hand front quarter of the room, (3) moving about continuously over the whole of the room area, and (4) sitting quietly throughout at the end of the room by the return grilles. The movement consisted of a steady walk at about 2 m.p.h. with frequent turns and reverses on moving in and out of the various areas delimited by the position of the vertical rods and other equipment.

Expression of the results

The underlying intention of these experiments was to investigate the extent to which two or more patients in bed in such a room would be effectively isolated from each other in spite of the disturbance introduced by the movements of nurses and others in the room. For this purpose, the beds would be placed with their head ends close to the inlet filter wall and their length parallel to the direction of air flow (see Fig. 3). The room was therefore considered as three areas: B 1 and B 2, the two quarters curtaining the beds; and R, the rear half of the room down-wind from these two. Dispersal took place either in the quarter B 1 at point A or in the rear half R at point D, and the contamination reaching the other bed area B 2 or both the bed areas B 1 and B 2 when dispersal took place in the rear half of the room was assessed by adding together the colonies found on all the twelve plates exposed in the relevant bed area. The number of spore-bearing particles discharged in a 20 min. test was $1{\cdot}6 \times 20 \times 10^6 = 3{\cdot}2 \times 10^7$ and the aggregate area of the twelve plates was 2 sq.ft. The total number collected represents therefore the average contamination within the area per square foot of surface for a dispersion of $6{\cdot}4 \times 10^7$ particles, and the results have been converted in the tables to the contamination per square foot per 10^8 particles dispersed. Since the particle-settling rate approximated to 1 ft./min., this is closely equivalent to the volumetric (e.g. inhaled) contamination rate per 10^8 particles dispersed per cubic foot of air per minute sampled or inhaled.

It is of interest to compare the results with those that would be expected if the ventilating air had been supplied at the same rate in such a way that the air was always completely mixed by turbulent motion in place of the directed air flow system. At the higher rates of air flow this would, of course, need intolerably high turbulent air velocities. Under these postulated conditions the rate of removal of dispersed particles would be exponential, and the total number that would settle on any square foot of horizontal exposed surface would be $(60NS)/VK$, where N is the total number of particles dispersed, S is the settling rate of the particles in feet

per minute, V is the volume of the room in cubic feet and K is the exponential die-away constant in hr.$^{-1}$.

K includes the effects of both ventilation and settling and is given by $60(v+SA)/V$, where v is the volume of ventilating air in cubic feet per minute and A is the total area of exposed horizontal surface in square feet. For the room and particles used in these experiments, $S = 1{\cdot}1$ ft./min., $V = 1360$ cu.ft. and $A = 167$ sq.ft. Hence $K = 0{\cdot}044v + 8$ and the calculated settling is

$$0{\cdot}049N(0{\cdot}044v+8) \text{ per sq.ft.}$$

RESULTS

Table 1 shows the results of the forty-eight individual experiments. The extent of transfer of air-dispersed contamination into the 'sensitive' area has been determined, as described in the previous section, as the number of particles settling per square foot of surface for 10^8 particles dispersed. The 'sensitive' area is defined as either both bed areas (B 1 and B 2), when dispersal took place at the rear of the room, or as the other bed area, B 2, when dispersal took place in bed area B 1.

Table 1. *Numbers of spore bearing particles settling per square foot of exposed surface in the 'sensitive' area for* 10^8 *particles dispersed: equivalent particle diameter* 13 μ

		Ventilation conditions					
		(a)	(b)	(c)	(d)	(e)	(f)
Linear air-flow velocity (ft./min.)		100	60	35	22	Turbulent	
Rate of air supply (cu.ft./min.)		8200	4900	2900	1800	1600	130
Ventilation rate/hr.		360	220	130	80	75	6
		Number of particles settling					
Calculated settling for turbulent ventilation		$1{\cdot}32 \times 10^4$	$2{\cdot}18 \times 10^4$	$3{\cdot}63 \times 10^4$	$5{\cdot}57 \times 10^4$	$5{\cdot}90 \times 10^4$	35×10^4
Group	Experimental conditions						
1	D 0-P, -C or -U	—	3, 10	—	—	—	—
	A 0-P, -C or -U	—	6, 0	25	0	$4{\cdot}0 \times 10^4$	15×10^4
	D 1-P, -C or -U	5, 3, 5, 10	—	13, 12	12, 207	$3{\cdot}2 \times 10^4$	29×10^4
	D 2-P	—	5	—	—	—	—
	D 2-C	2	8	—	—	—	—
	A 1-P	2	2	—	—	—	—
	A 2-P	7	0	10	2	—	—
2	D 3-P, -C or -U	40, 90 87, 65	—	255, 146, 104 222, 62, 85	590, 287, 500 900, 845, 557	—	—
	A 2-C	100	47	57	600	—	—
	A 3-P	149	—	119	445	—	—
3	A 2-U	202	—	650	7200	—	—
	A 3-U	162	380	1230, 1380	4150	$4{\cdot}1 \times 10^4$	34×10^4

The experimental conditions have been coded as follows. D, Dispersal at the rear of the room, at point D; A, dispersal in one bed area, B 1, at point A (at the front of the room). 0, No movement during dispersal; 1, movement in the rear half of the room only; 2, movement in the rear half of the room and into the non-sensitive bed area only; 3, movement over the whole area of the room; P, a solid partition separated the two bed areas; C, bed areas were separated by a curtain; U, Communication between the two bed areas was unobstructed.

When dispersal was at point D the 'sensitive' area comprised both bed areas, B 1 and B 2. When dispersal took place at point A, only the area B 2 formed the 'sensitive' area. The contamination level as determined in control exposures without dispersal was equivalent to eight particles settling in any test.

The numbers of spore-bearing particles recovered during the experiments with the two turbulent ventilating systems correspond reasonably closely to those calculated. With the directed flow system they are always much lower than this even at the lowest rate of air flow. The results obtained with the directed air-flow system fall into three clearly distinguishable groups according to the position of dispersal, extent of movement in the room during the particle dispersal and the presence or absence of the curtain or partition.

The first group comprises all those experiments with no movement of persons, those where dispersal took place at the rear of the room and movement was confined to the rear half of the room or movement extended into one of the bed areas only and this area was separated from the sensitive area either by a curtain or by a solid partition, together with those where dispersal took place in one bed area and movement was confined to the rear of the room or extended into the dispersal area and this area was separated from the sensitive area by a solid partition. With the exception of one experiment at the lowest air velocity (22 ft./min.) the numbers recovered do not significantly exceed the expected accidental contamination level due to manipulation obtained in control experiments; that is, contamination by settling does not exceed 10/sq.ft./10^8 particles dispersed and is probably much less. This is more than 1000 times less than the level that would be expected if the air in the room had been turbulently mixed. In this group, any contamination would have had to travel against the direction of air flow beyond the areas directly disturbed by movement of persons.

The second group showed contamination levels between about 100 and 1000 times less than those expected in a room where the air was turbulently mixed. This group comprises those experiments where dispersal took place at the rear of the room but movement of persons extended over the whole of the room, those where dispersal took place in one bed area and movement extended over the whole of the room but the sensitive area was separated from the dispersal area by a solid partition, and in addition those where dispersal took place in one bed area, movement was confined to this area and the rear of the room and the dispersal area was separated from the bed area by a curtain. In this group contamination had moved upstream against the direction of air flow in association with the movement of persons or had moved across the direction of air flow under the lower edge of the curtain when this was disturbed by persons moving alongside it. The colonies of *B. subtilis* recovered in these last experiments were confined to those plates placed on the floor within 1 ft. of the lower edge of the curtain. In this group the contamination increased as the velocity of air movement was reduced, but not significantly more than in proportion to the reduction in air velocity.

The third group includes the remaining experiments where the particles were dispersed in one bed area; there was neither curtain nor partition between this and the sensitive area and movement was either over the whole of the room or over the rear of the room together with the dispersal area. The contamination levels varied between 1/10 and 1/100 of those expected in a fully turbulently mixed room and increased more than proportionately to any reduction in the linear air velocity. In this group the contamination moved across the direction of air flow as a result of the movement of persons in the room.

The details of this process were examined in more detail by considering the colonies recovered on the plates exposed in the sensitive area in two groups: those recovered on the six plates exposed near to the centre line of the room, and those recovered on the six plates near to the wall and remote from the point of dispersal. The results of this analysis are given in Fig. 4. This shows, as would be expected, that many more colonies were found on those plates exposed near to the centre line. These were exposed little over 3 ft. from the line of particle dispersal and the number of colonies found depended little on whether or not movement of persons extend right over the sensitive area. Movement within the dispersal area in any

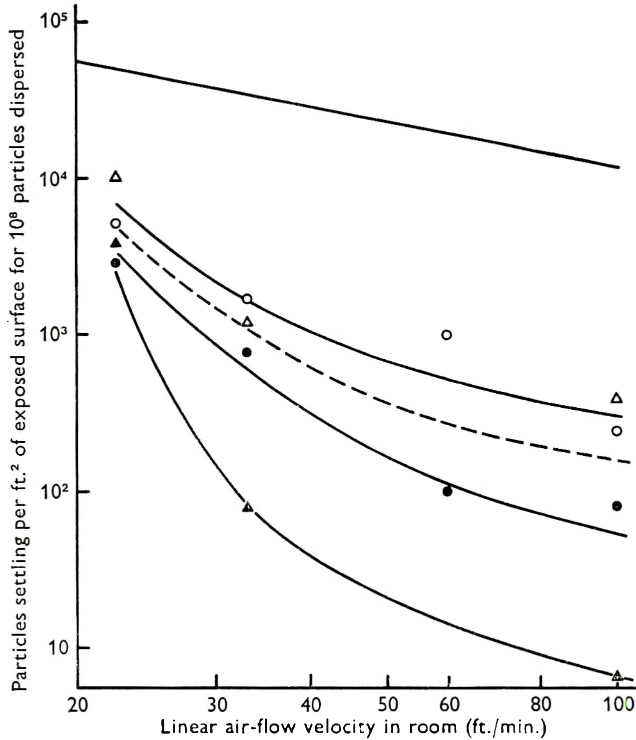


Fig. 4. Transfer across the direction of air flow. Dispersal took place at point A in area B 1. Triangular sumbols, △ and ▲, record experiments in which movement was restricted to the rear and area B 1 only (coded 2 in text and Table 1). Circular symbols, ○ and ●, record experiments in which movement extended over the whole of the room area (coded 3 in text and Table 1). Open symbols, △ and ○, record the spore-bearing particles recovered from the six plates exposed in area B 2, close to the centre-line of the room. Filled symbols, ▲ and ●, record the spore-bearing particles recovered from the remaining six plates exposed in area B 2 close to the wall of the room. The upper straight line shows the numbers of spore-bearing particles that would have been recovered if the air had been completely mixed by turbulent movements. The lower curves have been drawn in by eye to illustrate the effect of the various conditions on the number of spore-bearing particles recovered.

case reached to within inches of these plates. If the air velocity was 35 ft./min. or over the numbers of particles reaching the plates exposed near to the room wall were between 10 and 100 times less than the number found on the plates near the

centre line when movement did not extend into the sensitive area, and between 2 and 10 times less when movement extended over this area. When the air velocity was reduced to only 22 ft./min., however, the numbers recovered differed little between the two positions whatever the nature of the movement during particle dispersal.

The effect of a curtain or partition and the distribution of contamination in the three room areas is shown in more detail in Table 2 for the experiments carried out at a linear air velocity of 35 ft./min. In particular, this includes the results of one experiment in which, although the two subjects walked over the whole area of the room, they restricted their movements as if a curtain or partition had been in place although no such barrier was in position. It is clear that this restriction had little if any effect on the figures obtained.

Table 2. *Numbers of spore-bearing particles settling per square foot of exposed surface for* 10^8 *particles dispersed*

			Number of particles settling in		
Group	Movement	Partition or curtain	Dispersal bed area, B 1	'Sensitive' bed area, B 2	Rear half of room, R
2	Rear of room	Neither	15,500	650	22,000
	and dispersal	Curtain	21,000	57	53,500
	bed area only	Partition	14,000	10	38,500
3	Over whole of	Neither	6,500	1230	36,000
	the room area	Neither*	12,500	1380	34,000
		Partition	15,500	119	43,500

Equivalent particle diameter, 13 μ. Linear air velocity 35 ft./min. Dispersal took place in one bed area, B 1.

* Although there was no partition or curtain in position during this experiment, the two subjects restricted their movements in the room as if such an obstruction had been there.

DISCUSSION

These experiments seem to establish to a high level of significance that little if any particulate contamination moves against the direction of air flow even when there is considerable movement of persons in the room. Persons moving from a contaminated region can, however, transport small numbers of particles in any direction. Lateral transport of particles across the direction of air flow, however, does not extend to any significant extent beyond a few feet from the area in which the movement is taking place so long as the velocity of linear air movement does not fall below 35 ft./min.

In setting up a room with a directed air flow for clinical studies it is convenient, especially in relation to controlling noise from the machinery, to use as low an air velocity as is compatible with efficiency. These experiments suggest that a horizontally directed air flow of 40 ft./min. with an inter-bed spacing of 5–6 ft. should be effective in eliminating airborne transfer of bacteria-carrying particles from one patient to another within the room. Protection from contamination generated at the down-stream end of the room should be even better. This ability to nurse more

than one patient in a single room and to allow unrestricted access to the down-stream end of the space without impairing isolation from airborne infection should substantially ease the nursing and supervisory difficulties associated with isolation procedures and reduce the psychological problems that an isolation regime may induce in patients. The extent to which air isolation is important in practice for any class of patient is by no means clear, and a principal objective in future studies carried out in this room will be an exploration of infection transferred via nursing procedures in an environment free from the risk of airborne infection.

SUMMARY

Experiments have been carried out on the extent to which movement of persons in a room ventilated by a horizontally directed uniform air velocity can transport airborne bacteria from one position to another. More than 10^7 particles approximately 13 μ in diameter (settling rate in air about 1 ft./min.) carrying spores of *Bacillus subtilis* var. *niger* were liberated in each experiment and the numbers reaching the different parts of the room were estimated by those recovered on exposed settling plates. At air velocities of 35 ft./min. and over, no particles could be certainly found to have moved against the direction of air flow except into areas actually entered by persons, and the numbers found in these areas were between 100 and 1000 times less than would have been expected in rooms turbulently ventilated with the same volume of air. There was some transport of particles transversely across the air-flow lines but at 4 ft. distance from the area where movement of persons was taking place the numbers had fallen substantially below 1/100 of those to be expected in a turbulently ventilated room.

The results recorded at an air velocity of 22 ft./min. were significantly less satisfactory.

Our thanks are due to the N.W. Metropolitan Regional Hospital Board, to the Hospital Management Committee of St Charles' Hospital, London, W10, and especially to the hospital engineer for accommodating the experimental room and facilitating its operation.

REFERENCES

BEEBY, M. M. & WHITEHOUSE, C. E. (1965). A bacterial spore test piece for the control of ethylene oxide sterilization. *J. appl. Bact.* **28**, 349.

LEVITAN, A. A., SEIDLER, F. M., STRONG, C. D. & HERMAN, L. G. (1968). The role of supportive services in the operation of an isolator system. *J. Am. med. Ass.* **203**, 1009.

LIDWELL, O. M., RICHARDS, I. D. G. & POLAKOFF, S. (1967). Comparison of three ventilating systems in an operating room. *J. Hyg., Camb.* **65**, 193.

MAY, K. R. (1949). An improved spinning top homogeneous spray apparatus. *J. appl. Phys.* **20**, 232.

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

J. Hyg., Camb. (1969), **67**, 107
Printed in Great Britain

Some factors affecting the viability of freeze-thawed T4 bacteriophage

By P. R. M. STEELE, J. D. DAVIES and R. I. N. GREAVES
The University Department of Pathology, Cambridge, England

(*Received* 15 *July* 1968)

INTRODUCTION

It was reported in a previous communication (Greaves, Davies & Steele, 1967) that the T4 bacteriophage of *Escherichia coli* was highly sensitive to both freeze-thawing and freeze-drying. The structure of this virus is known in considerable detail and its viability can be easily and accurately determined. Therefore it seemed to be a suitable model for investigation into injury by freezing followed by thawing or drying, and the mechanism of action of protective additives.

This paper reports some effects of freeze-thawing on purified T4 phage, and a preliminary investigation into the nature of the protection afforded by peptone, a product which has been widely used as a protective against freeze-thawing and freeze-drying injury.

MATERIALS AND METHODS

Host bacteria

The host organism *Eschericha coli* B was grown in nutrient broth (Hartley's tryptic digest broth pH 7·4) at 37° C. in 6 ml. volumes for titre determination, or in 500 ml. volumes with aeration for phage preparation.

Bacteriophage

The T4 phage were prepared from a 500 ml. lysed culture of *E. coli* B. Bacterial debris was removed by centrifugation at 7500*g* for 30 min. The supernatant was centrifuged at 30,000*g* for 60 min., and the pellet resuspended at 4 °C. overnight in 0·13 M phosphate buffer (KH_2PO_4-Na_2HPO_4), with 2 μg./ml. of DNA-ase. The T4 phage were purified by two further cycles of low- and high-speed centrifugation. The final translucent pellet was resuspended in 0·13 M phosphate buffer. Purified phage stocks were stored at 4° C. The phage titres were determined by the standard top-agar technique of Adams (1959): phage samples were mixed with bacteria in 2·5 ml. liquefied agar at 44° C., and the mixtures poured as a thin layer onto agar plates. The plates were incubated overnight at 37° C. and the next day the bacteriophage plaques in the bacterial lawn were counted. Duplicate samples, each plated on three separate plates, were used for every experimental determination of viability.

Freezing and thawing procedure

Experimental samples, consisting of 0·1 ml. T4 phage suspended in 0·13 M phosphate buffer or 0·13 M phosphate buffer plus additive were measured into glass

freeze-drying tubes 10 cm. in length and 5 mm. internal diameter (Johnson and Jorgensen Ltd.). Unless otherwise stated, the concentration of T4 phage in the experimental samples was 1–2 × 10^6 p.f.u./ml. The samples were cooled at 1° C./min. on the freezing stage of the experimental freeze-drying unit previously described by Greaves & Davies (1965). At −5° C. freezing was induced by touching the surface of the sample with a fine wire cooled in liquid nitrogen. Rapid thawing was achieved by shaking the frozen sample in a 37° C. water bath. Samples were thawed slowly by placing them in a thick block of polystyrene maintained at 4° C.

Peptone

The peptone used in this investigation was a papaine digest of muscle ('Bacteriological Peptone', Evans Ltd.).

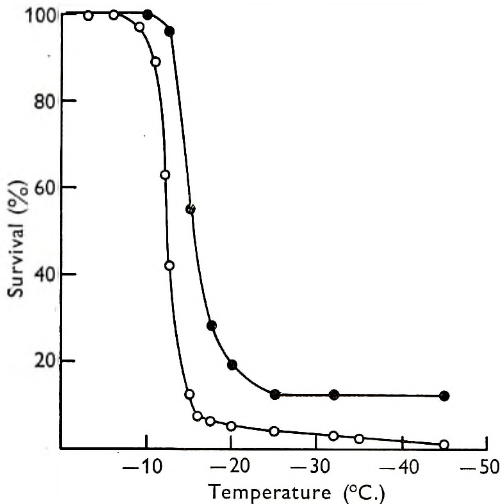


Fig. 1

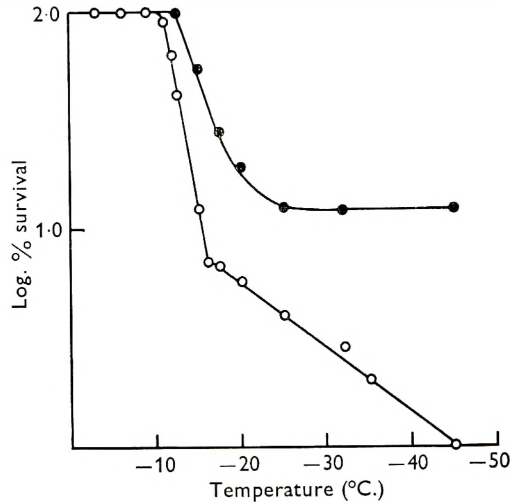


Fig. 2

Fig. 1. The percentage survival of frozen-thawed T 4 phage suspended in 0·13 M phosphate buffer. Samples were cooled at 1° C./min. to the indicated temperatures and thawed either rapidly (○) or slowly (●).

Fig. 2. The logarithmic plot of the results shown in Fig. 1. The rapidly thawed T 4 phage appear to be inactivated by two separate processes.

RESULTS

Effect of the suspending medium

Phosphate buffer

Samples of T4 phage suspended in 0·13 M phosphate buffer were cooled at 1° C./min. to temperatures from 0° to −45° C., and thawed either rapidly or slowly, and the percentage survival determined (Fig. 1). The viability of rapidly thawed phage fell sharply between the freezing temperatures of −11° and −15° C. to 10 % survival. Thereafter the viability fell slowly to 1 % survival following rapid thawing from −45° C. The logarithmic plot of the results (Fig. 2) suggests that the rapid-thaw inactivation results from two separate processes. The inactivation of slowly thawed T4 phage occurred between −10° and −25° C.

Addition of sodium chloride

Sodium chloride was added to experimental samples in concentrations of 0·15 M and 0·5 M. Despite the large difference in initial molarity both concentrations of NaCl had a similar effect on the viability of freeze-thawed phage (Fig. 3). Most of the inactivation of rapidly thawed samples occurred in a temperature range 3° C. higher

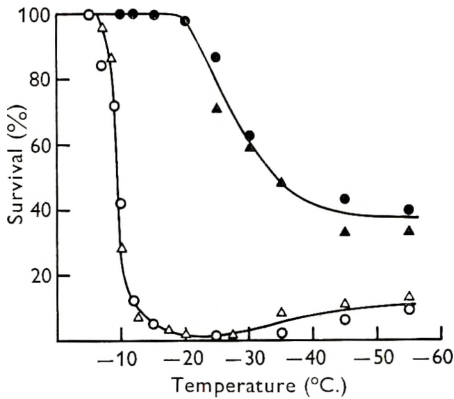


Fig. 3

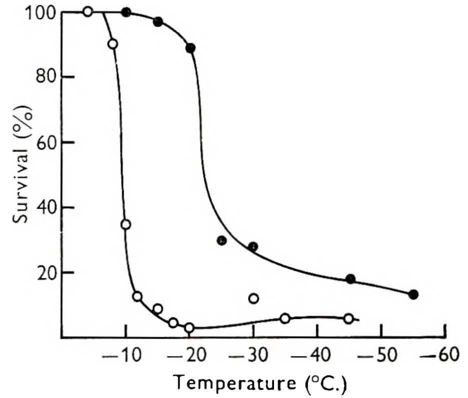


Fig. 4

Fig. 3. The percentage survival of frozen-thawed T 4 phage suspended in 0·13 M phosphate buffer + NaCl. Samples were cooled at 1° C./min. to the indicated temperatures. ○, Buffer + 0·15 M-NaCl, rapid thaw; ●, buffer + 0·15 M-NaCl, slow thaw; △, buffer + 0·5 M-NaCl, rapid thaw; ▲, buffer + 0·5 M-NaCl, slow thaw.

Fig. 4. The percentage survival of frozen-thawed T 4 phage suspended in 0·13 M phosphate buffer + 0·15 M-KCl. Samples were cooled at 1° C./min. and thawed either rapidly (○) or slowly (●).

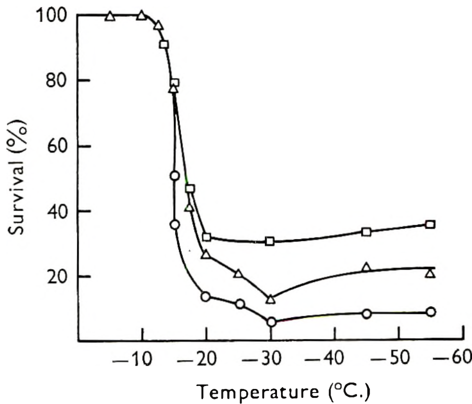


Fig. 5

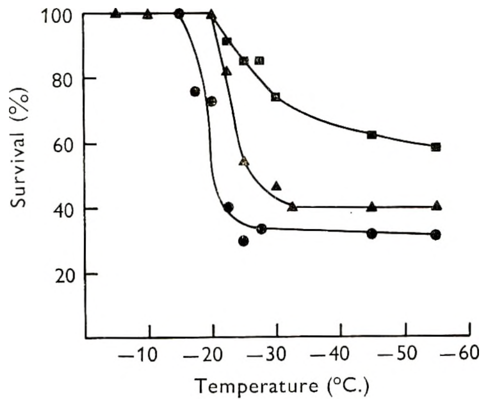


Fig. 6

Fig. 5. The effect of addition of peptone on the survival of frozen-thawed T 4 phage. Samples were cooled at 1° C./min. to the indicated temperatures and thawed rapidly. The concentrations (w/v) of added peptone were: ○, 1 %; △, 5 %; □, 10 %.

Fig. 6. The effect of addition of peptone on the survival of frozen-thawed T 4 phage. Samples were cooled at 1° C./min. to the indicated temperatures and thawed slowly. The concentrations (w/v) of added peptone were: ●, 1 %; ▲, 5 %; ■, 10 %.

than with buffer alone, but the gradient of the viability curve was identical. Between − 15° C. and − 25° C. there was a dip in the viability curve. The presence of NaCl greatly increased the survival of slowly thawed T4 phage, inactivation occurring between − 20° and − 45° C.

Table 1. *The effect of amino acids at a concentration of* 0·1 M *on the survival of T 4 phage cooled at* 1° *C./min. to* − 45° *C. and thawed either rapidly* (*RT*) *or slowly* (*ST*)

Amino acid	Survival RT (%)	Survival ST (%)	Amino acid	Survival RT (%)	Survival ST (%)
(Buffer)	1·0	10	Methionine	1·0	15
Glycine	1·0	14·8	Phenylalanine	0·5	—
Alanine	2·8	9·4	Histidine	0·8	22
Valine	3·2	5·8	Arginine	0·2	1·7
Leucine	1·3	15·5	Lysine	2·4	1·3
Iso-leucine	1·0	14·5	Aspartic acid	3·7	8·8
Serine	1·0	25	Glutamic acid	4·0	22
Threonine	0·5	12	Proline	0·7	6·2
Cystine	1·2	—			

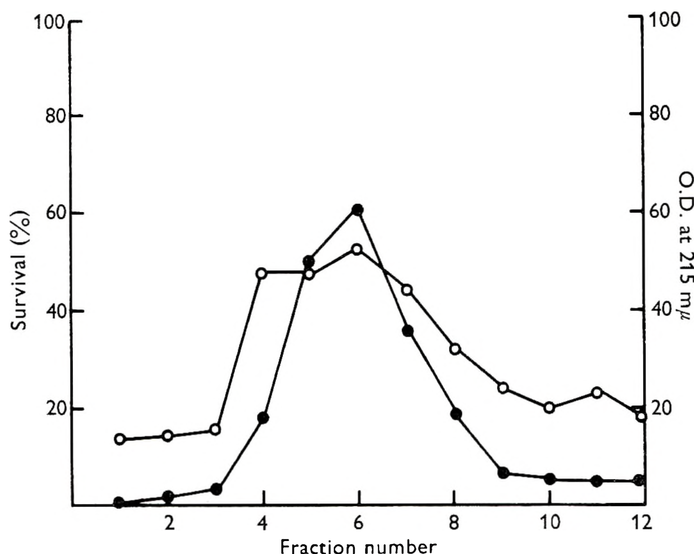


Fig. 7. The effect of G 25 Sephadex fractions of peptone on the percentage survival T 4 phage cooled at 1° C./min. to − 45° C. and thawed slowly. The phage were suspended in 0·13 M phosphate buffer + peptone fraction. For details of fractionation procedure see text. ○, % survival; ●, O.D.

Addition of potassium chloride

The effect of added 0·15 M-KCl on the viability of freeze-thawed phage (Fig. 4) was similar to that observed with NaCl. The survival of slowly thawed phage was increased, but to a lesser degree than with NaCl, most of the inactivation occurring between − 15° and − 35° C.

Effect of peptone

Peptone was added to T4 phage samples in concentrations of 1 %, 5 % and 10 % (w/v). The addition of peptone progressively increased the survival of rapidly

thawed phage, although the gradient of the inactivation curve was not appreciably altered (Fig. 5). The temperature range of inactivation was lowered by 2–5° C.

Peptone gave significant protection to slowly thawed phage (Fig. 6), markedly altering both the gradient and temperature range of inactivation.

Amino acids

Separation of a 1 % solution of peptone by two-dimensional paper electrophoresis showed that it contained most of the common amino acids together with small peptides of all charges. Since there were so many amino acids present in peptone, pure amino acids were tested separately for protection against freeze-thaw injury at

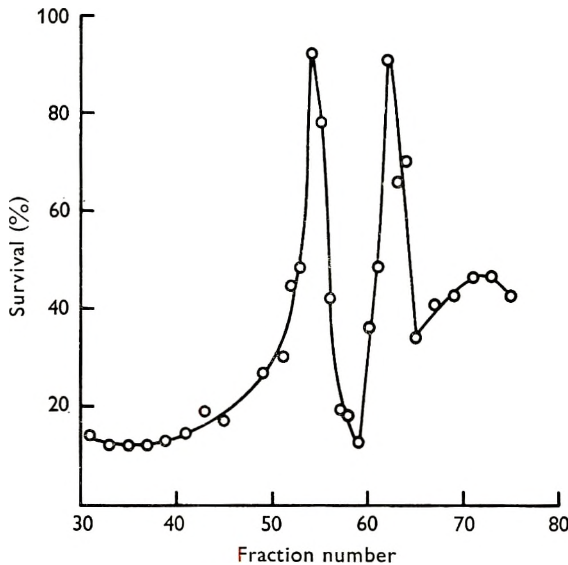


Fig. 8. The effect of twice-fractionated peptone. T 4 phage suspended in 0·13 M phosphate buffer + peptone fraction were cooled at 1° C./min. to −45° C. and thawed slowly. There are two well-defined protective peaks.

a concentration of 0·1 M (Table 1). None of them had a significant effect on rapidly thawed T4 phage. There was some variation in their effect on slowly thawed samples: arginine and lysine decreased survival, whereas serine, histidine and glutamic acid were slightly protective. However, there was no indication that the significant protection afforded by peptone was due to its amino-acid constituents.

Sephadex fractionation

A preliminary separation of peptone was carried out by fractionating 20 mg. of peptone through a 40 cm. column of G25 Sephadex (50 ml. bed-volume). The resulting 5 ml. fractions were concentrated five-fold by freeze-drying and tested for protective effect (Fig. 7). The position and relative concentration of peptide were estimated by absorption at 215 mμ. The middle fractions gave good protection to slowly thawed T4 phage, moreover the maximum survival was increased by fractionation. The fractions had no significant effect on the viability of rapidly thawed phage.

Following this preliminary experiment, 800 mg. of peptone was fractionated through a wide G25 Sephadex column; the middle peptide fraction was collected and freeze-dried. The resulting 450 mg. of material was refractionated through a

Table 2. *The effect of initial T4 phage concentration on survival*

(The samples were cooled at 1° C/min. to −45° C. and thawed either rapidly or slowly.)

Suspending medium	Initial conc. of phage p.f.u./ml.	Survival rapid thaw (%)	Survival slow thaw (%)
0·13 M buffer	$3·7 \times 10^8$	1·2	44
	$1·2 \times 10^8$	1·3	33·5
	$1·2 \times 10^7$	2·6	12
	$1·2 \times 10^6$	1·5	13
	$1·2 \times 10^4$	1·8	11·7
0·13 M buffer + 0·15 M-NaCl	$3·9 \times 10^8$	15·5	82·5
	$1·3 \times 10^8$	14·4	74
	$1·3 \times 10^7$	11·7	60
	$1·3 \times 10^6$	13·4	41

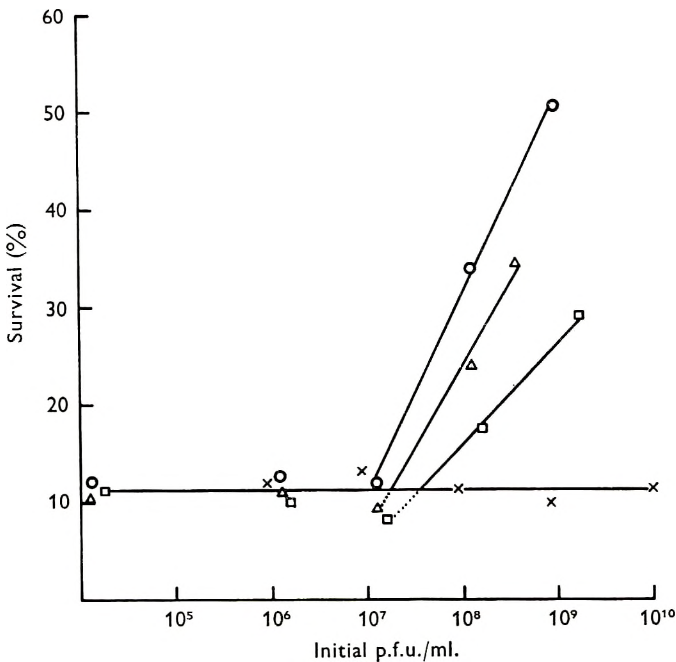


Fig. 9. The effect of initial phage concentration and 'washing' on the survival of T 4 phage cooled at 1° C./min. to −45° C. and thawed slowly. The suspending medium was 0·13 M phosphate buffer. T 4 phage purified from broth lysates of *Escherichia coli* B were washed with distilled water while adsorbed on to magnesium pyrophosphate gel. This treatment considerably lowered the high survival obtained with concentrated suspensions. No concentration effect was observed with phage purified from a lysate of *E. coli* B grown in Adams' medium. ○, Washed 3 times; △, washed 7 times; □, washed 12 times; ×, phage prepared from a lysate of *E. coli* B grown in Adams' medium.

160 cm. G 25 Sephadex column (800 ml. bed-volume) and the 10 ml. fractions concentrated tenfold by freeze-drying and tested for protective effect, against inactivation of slowly thawed T 4 phage (Fig. 8). There were two well-defined peaks of protection with maxima of 90 % survival, followed by a broad peak with a maximum of 45 % survival.

Unfortunately further investigation of the protective fractions proved to be very difficult. Electrophoretic separation of the fractions at pH 3·5 showed that they contained a large range of peptides which did not fall into discrete bands.

Effect of the initial T 4 phage concentration

The initial phage titre routinely used in experimental samples was $1\text{–}2 \times 10^6$ p.f.u./ml. When higher concentrations of T 4 phage were freeze-thawed a marked concentration effect was observed (Table 2); the survival of slowly thawed phage was considerably increased, although no such effect was observed with rapidly thawed samples: possibly the phage had absorbed protective compounds from the original broth lysate. As a test of this, the phage were adsorbed on to magnesium pyrophosphate gel (Schito, 1967) and washed several times with distilled water, before being eluted off the gel with 0·13 M phosphate buffer. The effect of washing is shown in Fig. 9. Clearly a large part of the protection can be 'washed off' the phage. As a further test, T 4 phage were prepared by lysing *E. coli* B grown in the defined salt medium of Adams (Adams, 1959) instead of in nutrient broth. These phage showed no increase in survival with increase in concentration.

DISCUSSION

It is significant that most of the inactivation of rapidly thawed T 4 phage occurred in a narrow temperature range, which was only slightly altered by addition of salts or peptone. Rapid thawing produces a sudden dilution of concentrated solutes and thus a sharp fall in osmotic pressure of the suspending medium. The T 4 phage used in the present experiments was inactivated to 1 % viability by rapid 100-fold dilution from 3 M-NaCl into distilled water, but remained 100 % viable if the dilution was carried out slowly. Anderson (1953) termed this phenomenon of inactivation caused by a rapid fall in salt concentration 'osmotic shock'. Slow thawing effectively produces a slow dilution of the solutes concentrated during freezing. Little inactivation of slow-thawed samples occurred in the temperature range in which most of the rapid-thaw inactivation was observed. It therefore seems likely that osmotic damage was responsible for most of the inactivation of rapidly thawed T 4 phage. Leibo & Mazur (1967) also implicated osmotic damage as a cause of inactivation in freeze-thawed T 4 phage.

The eutectic temperatures of the suspending media used in the experiments are: phosphate buffer −4·5° C.; buffer + NaCl −23·5° C.; buffer + KCl −12·5° C. (Van den Berg, 1959). Slowly thawed T 4 phage were inactivated only below the eutectic temperature of the respective suspending medium. Furthermore viability was increased by lowering the eutectic temperature. This relationship between eutectic temperature and freeze-thaw damage indicates that the damage is due to removal of

the last traces of unbound water as ice, and that damage is most severe when this water is removed at a relatively high freezing temperature.

Peptone strongly protected against inactivation of slowly thawed T 4 phage. The results obtained by fractionation of peptone showed that the constituents largely responsible for this protection could be separated as two peptide fractions. Unfortunately the unspecific degradation of protein by papaine produces a range of innumerable small peptides and amino acids. Such a mixture is clearly unsuitable for future investigation into the nature of the protection afforded by peptides. What is required is a specific enzymic digest of a pure protein of known amino acid sequence. Work along these lines is now in progress using a tryptic digest of rabbit globin.

It was shown in the experiments that the increase in survival of slow-thawed T 4 phage produced by increasing the initial phage titre was greatly diminished by washing the phage with distilled water, and was entirely prevented by preparing the T 4 phage stock in a salt medium instead of nutrient broth. In the light of the results obtained with the peptide fractions of peptone it seems possible that the concentration effect may have been caused by protective peptides adsorbed on to the phage from the original broth lysate.

SUMMARY

Some effects of freeze-thawing on the T 4 bacteriophage have been studied. The results indicated that most of the inactivation of rapidly thawed samples was due to osmotic damage, whereas inactivation of slowly thawed samples appeared to be correlated with the eutectic temperature of the suspending medium. Peptone significantly increased the survival of slowly thawed T 4 phage. The particular constituents of peptone largely responsible for this protection were separated as two distinct peptide fractions, using G 25 Sephadex.

This work has been supported in part by grants from the Medical Research Council and the Office of Naval Research under contract F61052-68-C-0041.

REFERENCES

Adams, M. (1959). *Bacteriophages*. New York: Wiley.

Anderson, T. F. (1953). The morphology and osmotic properties of bacteriophage systems. *Cold Spring Harb. Symp. quant. Biol.* **18**, 197–206.

Greaves, R. I. N. & Davies, J. D. (1965). Separate effects of freezing, thawing and drying living cells. *Ann. N.Y. Acad. Sci.* **125**, 548–58.

Greaves, R. I. N., Davies, J. D. & Steele, P. R. M. (1967). The freeze-drying of frost-sensitive organisms. *Cryobiology* **3**, 283–7.

Leibo, S. B. & Mazur, P. (1967). Freezing of bacteriophage T4B. *Cryobiology* **3**, 366–7.

Schito, G. C. (1967). A rapid purification procedure for bacterial viruses. *Virology* **30**, 305–7.

Van den Berg, L. (1959). Effect of additions of NaCl and KCl on the system of KH_2PO_4-Na_2HPO_4-H_2O on pH and composition during freezing. *Archs Biochem. Biophys.* **34**, 305–15.

J. Hyg., Camb. (1969), **67**, 115
With 4 plates
Printed in Great Britain

The development of feline cell lines for the growth of feline infectious enteritis (panleucopaenia) virus

BY K. J. O'REILLY AND A. M. WHITAKER
Wellcome Research Laboratories, Beckenham, Kent

(*Received* 25 *July* 1968)

The first published account of the successful isolation of feline infectious enteritis (FIE) virus in tissue culture was by Johnson (1964). He used primary kitten kidney tissue cultures and relied upon staining with haematoxylin and eosin (H and E) for the initial recognition of virus isolation and for subsequent studies on intranuclear changes in affected cells (Johnson, 1965). Other workers have also used primary kitten kidney tissue culture in studies of FIE virus (King & Croghan, 1965; Gorham *et al.* 1965).

In these laboratories a fairly common finding has been the presence of wild FIE virus in primary kitten kidney tissue cultures, a fact which did not become evident in uninoculated control cultures until the seventh day after seeding. Since cultures are normally inoculated with virus on the second or third day after seeding, when the cell sheet is three-quarters confluent, and harvesting takes place 2 or 3 days later, warning of contamination with wild virus by its effect on control cultures comes too late. Apart from the interruption to research work, the possible presence of wild virus in primary kitten kidney tissue cultures is clearly a serious hazard in any attempt to develop a modified vaccine virus by serial passage in this tissue.

The cost of rearing cats in complete isolation for the provision of a regular supply of susceptible kidney tissue is prohibitive. Accordingly, the development of feline embryonic cell lines for the study of FIE virus was undertaken, and this also provided an opportunity for a detailed investigation of the chromosomes of the domestic cat.

MATERIALS AND METHODS

Cell culture

Feline embryos were removed by Caesarian section from normal healthy pregnant queens and washed in sterile phosphate buffered saline (PBS) containing 200 units of penicillin and 100 μg. of streptomycin per ml. After removal of the embryos from their membranes, various organs were taken and chopped into very small fragments. The tissue was trypsinized as described by Dulbecco & Vogt (1954) and the resulting suspension seeded at the rate of 5×10^6 cells per 4 oz. medical flat containing 10·0 ml. of medium. The medium consisted of 80 % Eagle's Medium Basal modified to have twice the usual amount of amino acids and vitamins, 10 % tryptose phosphate broth, 10 % adult bovine uninactivated serum, 200 units of penicillin and 100 μg. of streptomycin per ml.

After 2–3 days incubation at 37 °C. a confluent monolayer developed which was

subcultured in the following manner. The medium was removed and the cells were gently washed for 30 sec. in 3·0 ml. of 0·1 % trypsin in 0·05 % versene. This was quickly followed by pouring off the trypsin–versene mixture and the bottles were placed cell-sheet down on the bench. Several minutes later the cells began to detach from the glass and the bottles were again shaken until all the cells were free. The cells were then resuspended in 5·0 ml. of medium and divided equally between two 4 oz. medical flats. Eight ml. of medium were added to each bottle, which was then incubated at 37° C.

Viruses

Although most studies were done with a strain (12B1) of FIE virus isolated from the kidneys of an infected cat, other strains recovered from infected spleens and intestines were used. A virus isolated from a leopard (Johnson, 1964) and a strain of mink enteritis virus (Johnson, 1967*a*) were also tested.

Virus propagation and assay

For the isolation of virus, infected tissues were ground in a mortar and a 1/10 suspension in PBS prepared. After centrifuging at 800 rev./min. for 5 min., tenfold dilutions of the supernatant were made and 0·2 ml. of each dilution inoculated into test-tubes containing coverslips and $2{\cdot}0 \times 10^5$ of freshly versenized cells in 2·0 ml. of medium. The tubes were incubated at 37° C. at an angle of about 10°. On the third day after infection the coverslips were fixed in Bouin's solution and stained with H and E. Evidence of viral infection was accepted when the typical intranuclear changes were observed.

Subsequent passaging of virus was done by inoculating 2·0 ml. of infected tissue culture cells and fluid into 4 oz. medical flats containing freshly versenized cells in 10·0 ml. of tissue culture medium. Periodically, coverslip preparations were infected to ensure that the virus was being passaged. Virus-infected cultures were frozen at −30° C. after 3 days incubation.

For virus titrations, 0·2 ml. of twofold dilutions in PBS were inoculated into test-tubes containing coverslips. Initially, coverslips from each dilution were removed daily over a period of 5 days, fixed in Bouin's solution and stained with H and E. Latterly, the coverslips from all dilutions were fixed and stained on the third day only after infection. The stained preparations were examined under a ×40 objective and the degree of infection was estimated by counting the proportion of cells showing intranuclear changes in five arbitrarily chosen microscope fields. On average, about 450 cells were counted on each coverslip. The percentage of infected cells was then calculated.

Chromosome preparations

Metaphase chromosome preparations were obtained using a modification of the Rothfels & Siminovitch (1958) technique. Actively growing cultures (usually on the second day) were incubated with 0·04 mg./ml. final concentration colcemide for 3–6 hr. The cells were detached from the glass using trypsin–versene mixture

and sedimented by centrifuging for 5 min. at 800 rev./min. The supernatant was discarded and the pellet resuspended in 0·5 ml. of calf serum. Two ml. of distilled water were added slowly, and after mixing the suspension was left standing for 10 min. It was then centrifuged for 5 min. at 800 rev./min. and 0·5 ml. of freshly prepared fixative (1 volume reagent grade glacial acetic acid and 3 volumes reagent grade ethyl alcohol) added, care being taken not to break-up the pellet. After 20–30 min. the cells were resuspended with a fine pasteur pipette, centrifuged, washed with two changes of fixative, and a drop placed on a clean slide. The slides were air-dried at 37° C. and stained with either Giemsa or Orcein.

RESULTS

Production of cell lines

Embryos from twenty-two pregnancies were used in fifty attempts to produce diploid cell lines from various tissues. In some cases the whole embryos were used, especially when they were 1·0 cm. or less in length; in other cases, portions of the embryos were used, i.e. heart, kidney, liver, lung, skin, small intestine, spleen, tongue, voluntary muscle or combinations of organs such as lung and heart, liver and small intestine, or eviscerated and decapitated carcases. On two occasions amnion was the source of cells.

Table 1. *Development of feline diploid cell lines and susceptibility to FIE virus*

Embryonic tissue	Growth vigour	Susceptibility to FIE virus
Lung	+	+
Lung and heart	+	+
Embryo whole carcase	+	+
Voluntary muscle	+	+
Kidney	+	+
Heart	+	+
Skin	+	+
Amnion	+	±
Small intestine	+	±
Small intestine and liver	+	±
Tongue	+	±
Liver	−	NT
Spleen	−	NT

+ = satisfactory, − = unsatisfactory, ± = Poor, NT = not tested.

Tissue cultures prepared from liver and spleen were very poor and never exceeded four passages. Heart cells grew satisfactorily but were less vigorous by comparison with other cells. Lung cells (FEL) and embryo cells (F Emb), whether or not the latter cells originated from whole or eviscerated and decapitated carcases, grew prolifically (Table 1). Accordingly, for reasons of convenience the work was largely devoted to these two types of cell lines. Of the first five cell lines produced, four became contaminated with mycoplasma and the fifth died at the 20th passage. Another nine attempts to propagate diploid cell lines failed because,

in seven cases the cells did not grow, and in the other two there was bacterial contamination. Eleven embryo and seven lung diploid cell lines exceeded more than five serial passages. One lung cell line, despite contamination with mycoplasma, was passaged more than 100 times and a lung and an embryo cell line from another foetus each exceeded 70 passages.

In general, tissues from embryos of 4·5 cm. or less in length were suitable for the production of diploid cell lines and 3·0–4·0 cm. appeared to be optimal for diploid lung cell lines (Table 2).

A number of cell lines were stored frozen in liquid nitrogen at −170° C. and have been successfully revived.

Table 2. *Relationship of feline embryo size and production of diploid cell lines*

Embryo size (cm.)	Proportions of litters producing cell lines exceeding 5 passages	Type of cell lines	
		Embryo	Lung
0·5	3/6	3	ND
1·0	2/4	2	ND
1·5	1/1	1	1
3·0	2/2	ND	2
4·0	5/6	4	3
4·5	1/1	1	1
Full term	0/2	0	0
Totals	14/22	11	7

ND = not done

Cell Morphology

After the first four or five passages all the diploid cell lines were clearly fibroblastic in morphology (Pl. 1, fig. 1.) and remained so for the next thirty passages. At this point it was noted that the time taken for the cultures to become fully confluent lengthened from 2–3 to 5–6 days. By the 45th passage the cells had regained their vigour and needed subculturing every 3 days. At about the same time they began to assume a polygonal shape (Pl. 1, fig. 2), which remained a dominant feature until about the 60th passage when a further change was noted. The cells now showed uneven distribution with foci of relatively few cells surrounded by dense areas of cells stacked in a disorganized arrangement (Pl. 1, fig. 3).

Karyology

The incidence of tetraploidy in cultures before the 35th passage was 106 (3.4 %) in 3150 metaphases examined. Of 364 nuclei counted accurately, 33 had less than 38 chromosomes; thus revealing 9 % subdiploidy (Table 3). Sixteen (4·4 %) gaps or breaks were detected. However, these deviations fall within the accepted limits for cultured human cells as laid down in the Revised Standards for Karyology of Human Diploid Cell Strains (1966).

Chromosomes from fifty metaphases were photographed, measured and two values calculated. The first, the arm-length ratio, is expressed as the length of the

longer arm relative to the shorter one. The second, the percentage mean index length, expresses the average length of a pair of chromosomes as a percentage of the sum of the average lengths of all the pairs of chromosomes. The chromosomes (Table 4) and the karyotype (Pl. 2, figs. 4, 5) are arranged to follow, in general, the recommendations of the Denver Conference on human chromosome karyotype (Book *et al.* 1960). The classification of chromosomes by Cranmore & Alpen (1964)

Table 3. *Chromosome frequency distribution in* 364 *cells*

Cells ...	1	4	8	20	328	2	2	1
Chromosomes	30	35	36	37	38	39	40	41

Table 4. *Quantitative characteristics of the feline mitotic chromosomes*

Group	Chromosome no.	Arm-length ratio	Percentage mean index length
A	1	1·9	9·7
	2	3·3	8·2
	3	3·0	6·2
	4	3·2	5·8
	5	3·4	5·5
B	6	1·1	8·7
	7	1·4	6·6
	8	1·3	6·0
	9	1·4	5·3
	10	1·4	4·8
	11	1·5	4·2
C (i)	12	1·2	3·8
	13	1·2	3·7
	14	1·0	3·3
C (ii)	15	∞	3·0
	16	∞	2·8
C (iii)	17	1·0	2·7
	18	1·0	2·3
Sex chromosomes	*X*	1·5	5·6
	Y	1·0	1·9

has been simplified into three main groups. Group A comprises five large sub-telocentric chromosomes having an arm-length ratio greater than 1·9. There are six large metacentric chromosomes in group B, whose arm-length ratios do not exceed 1·6. Group C contains the smaller chromosomes, whose percentage mean index lengths are less than 4·0. This group can be divided into three subgroups. Chromosomes 12, 13 and 14 are difficult to identify individually, except when the satellites on chromosome 14 are visible. Chromosomes 15 and 16 are telocentric and 17 and 18 are small median metacentric. The *X* chromosome is difficult to distinguish from numbers 8, 9 and 10, but the *Y* chromosome is easier, because it is the smallest and is metacentric.

Between the 40th and 50th cell passages significant deviations in the karyotype were noted. The modal chromosome number of a cell line derived from the whole

embryo dropped from 38 to 37, and by the 55th passage 73% of the nuclei examined had 37 chromosomes (Pl. 2, fig. 6), while cell lines derived from lung tissue showed more striking deviations from the normal. Among translocations affecting a large number of chromosomes, ring formation of chromosome 6 and translocation of part or all of small chromosomes on to the number 2 chromosome were frequently observed (Pl. 2, fig. 7). In one 48th passage culture only 10% of the nuclei examined appeared to have a normal chromosome complement.

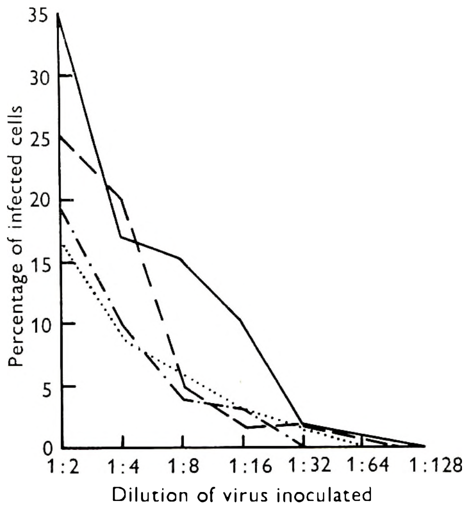


Fig. 1

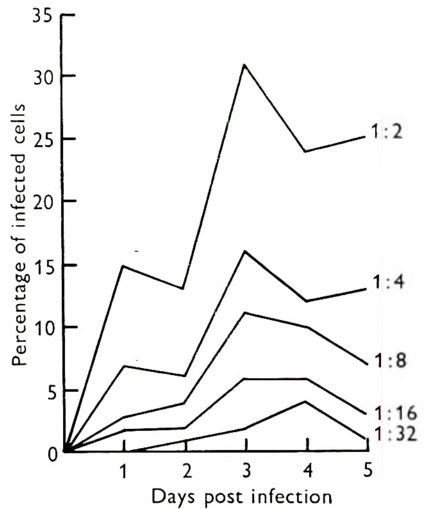


Fig. 2

Text-fig. 1. Percentage of cells infected with dilutions of 15th passage of FIE virus 72 hr. after inoculation of three feline embryo diploid cell lines. ——, 4th passage of embryo 17 cell line. — —, 10th passage of embryo 17 cell line. —·—, 8th passage of embryo 20 cell line. .... 3rd passage of embryo 21 cell line.

Text-fig. 2. Growth curves of dilutions of FIE virus in 45th passage of feline embryo lung cell line.

Virus susceptibility

All the cell lines were tested for susceptibility to FIE virus. Only one, FEL 1A, was insusceptible, probably because it was contaminated with mycoplasma. Five different FIE viral isolates as well as the leopard and mink enteritis viruses were grown and passaged and one FIE virus strain (12BI) was serially passaged more than 25 times. The passage level of the cells tested ranged from the 2nd to the 70th. Some variation in viral susceptibility between cell lines of different tissues and between cell lines of different embryos was observed (Table 1; Text-figs. 1, 2).

The intranuclear changes in these cell lines following virus infection resembled the changes described by Johnson (1965) in primary kitten kidney monolayers. The earliest nuclear changes appeared about 16 hr after infection, with a peak of infection 48 hr. later (Pl. 3, figs. 8, 9). A CPE in unstained preparations was only observed, and then inconsistently, when large inocula of undiluted virus were used (Pl. 4, figs. 10, 11).

Effect of Mycoplasma *on viral susceptibility*

The first cell line (FEL 1A) tested for susceptibility to virus infection became contaminated with *Mycoplasma hominis*. This fact was not evident initially, and numerous unsuccessful attempts were made to passage the virus serially in these cells used between the 21st and 100th passages. The first passage of virus produced cellular infection on 21 of 23 (91 %) occasions. The infection rates at the second and third serial passages of virus were 71 % and 25 % respectively. In no instance was there any evidence of infection at the fourth and fifth passages. Proof that infection of the cells occurred at the first virus passages was obtained by observing the characteristic intranuclear changes in monolayer cultures stained by H and E, the development of a positive Feulgen reaction, and the specific staining of the perinuclear haloes with fluorescent labelled FIE antiserum.

The virus survived five passages when the cells were alternately primary kitten kidney cells and FEL 1A cells. During the next three alternate passages no virus was detected.

In three of the next four diploid cell lines developed, mycoplasma contamination was detected at the 12th passage of two of them and at the eighth passage of the third. These cells in their early passages yielded infective virus, but after a few passages in a laboratory adjacent to a mycoplasma infected area they became incapable of producing fully competent new virus.

Although there are no reliable methods of eliminating *Mycoplasma* from tissue cultures, contaminated virus suspensions were freed of the organism by heating the suspensions at 50° C. for 30 min.

DISCUSSION

Because primary kitten kidney cells may be infected with wild FIE virus it was necessary to search for a virus-free source of tissue culture. In this laboratory it did not prove possible to passage FIE virus serially in the following tissue culture cells: BHK, calf bone marrow, calf buffy layer, calf thyroid, chick embryo, dog kidney, HeLa, patas and pig kidney.

Both the lung and embryo cell lines were susceptible to infection with FIE virus. A comparison of the percentage cellular infection recorded in this paper with those of Johnson (1967*b*), who used primary kitten kidney monolayers, suggests that the cell lines are possibly more susceptible to infection than primary tissue.

Contamination of the cells with *Mycoplasma* interfered with the production of infective virus. Since no mycoplasmas were recovered from very low passages of any of the cell lines, the inference must be that the contamination was of laboratory origin. Strict application of good tissue culture technique and the exclusion from the laboratory of all other tissue culture systems have prevented a repetition of the earlier experiences. Similar interference with virus replication in tissue cultures contaminated by *Mycoplasma* has been reported to occur with adenovirus type 2, Rous sarcoma virus and Rous associated virus (Rouse, Bonifas & Schlesinger, 1963; Somerson & Cook, 1965). Viral infection in the second and third passages in FEL 1A cells may have been due to residual virus in the inoculum used

at the first passage, since we have found that FIE virus will withstand 70° C. for 30 min.

Recent publications by Nakanishi (1960), Matano (1963) and Cranmore & Alpen (1964) show disagreement in the karyotype of the domestic cat. Identification of individual chromosomes from the karyotypes of these workers was difficult and in no case were detailed measurements given. The simplified karyotype presented here, together with the calculation of arm-length ratio and percentage mean index length, should go some way to improving the position.

The significance of the abnormal karyotypes described is not obvious, but transformation to an established cell line results in several different chromosomal abnormalities. These findings support the theory of Sandberg (1966) that transformation occurs when cells break free from the normal genetic control and that changes in karyotype are a mere epiphenomenon of abnormal growth rather than its cause.

SUMMARY

Primary kitten kidney cultures are frequently contaminated with wild feline infectious enteritis (FIE) virus and this led the authors to develop feline embryo diploid cell lines. Monolayer cultures were prepared from the lungs or from eviscerated and decapitated carcases of embryos obtained by Caesarian section from healthy pregnant queens. At about the 30th passage, these cells lost their fibroblastic morphology to become polygonal. After a further thirty passages the monolayers exhibited foci of low cell density circumscribed by bands of cells stacked in disorganized arrangement. All the developed cell lines were susceptible to infection with FIE virus and produced intranuclear changes resembling those described by Johnson (1965) in primary kitten kidney monolayers.

On four occasions the cell lines became contaminated with *Mycoplasma* and although there was evidence that the virus could infect the cells, there was no production of infective virus.

A simple karyotype was devised in which the 38 chromosomes were arranged in three groups according to the arm-length ratio and the percentage mean index length. After the 50th passage many of the nuclei of lung-derived cultures exhibited abnormal chromosomes resulting from ring formation or translocation, whilst those of embryo culture demonstrated a new modal chromosome number of 37.

We are grateful to Mrs L. Hitchcock and Mrs P. Waller, A.I.S.T., for technical assistance, to Mr E. A. Jones, A.I.I.P., for the photomicrographs and to Dr R. H. Johnson, University of Bristol, for supplying the leopard and mink enteritis viruses.

REFERENCES

BOOK, J. A. AND OTHERS (1960). A proposed standard system of nomenclature of human metotic chromosomes. *Lancet* i, 1063.

CRANMORE, D. & ALPEN, E. L. (1964). Chromosomes of the domestic cat. *Nature, Lond.* **204**, 99.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167.

GORHAM, J. R., HARTSOUGH, G. R., BURGER, D., LUST, S. & SATO, N. (1965). The preliminary use of attenuated feline panleukopenia virus to protect cats against panleukopenia and mink against enteritis. *Cornell Vet.* **55**, 559.

JOHNSON, R. H. (1964). Isolation of a virus from a condition simulating feline panleucopaenia in a leopard. *Vet. Rec.* **76**, 1008.

JOHNSON, R. H. (1965). Feline panleucopaenia virus. II. Some features of the cytopathic effects in feline kidney monolayers. *Res. vet. Sci.* **6**, 472.

JOHNSON, R. H. (1967*a*). Feline panleucopaenia virus—*in vitro* comparison of strains with a mink enteritis virus. *J. small Anim. Pract.* **8**, 319.

JOHNSON, R. H. (1967*b*). Feline panleucopaenia virus. IV. Methods for obtaining reproducible *in vitro* results. *Res. vet. Sci.* 8, 256.

KING, D. A. & CROGHAN, D. L. (1965). Immunofluorescence of feline panleucopaenia virus in cell culture: determination of immunological status of felines by serum neutralization. *Can. J. comp. Med.* **29**, 85.

MATANO, Y. (1963). A study of the chromosomes in the cat. *Jap. J. Genet.* **38**, 147.

NAKANISHI, Y. H. (1960). Development of a kitten lung cell strain and its chromosomes. *Z. Zellforsch. mikrosk. Anat.* **51**, 138.

REVISED STANDARDS FOR KARYOLOGY OF HUMAN DIPLOID CELL STRAINS (1966). *IIIrd Annual Meeting of the Permanent Section on Microbiological Standardisation of the International Association of Microbiological Societies*, Philadelphia.

ROTHFELS, K. H. & SIMINOVITCH, L. (1958). An air drying technique for flattening chromosomes in mammalian cells grown *in vitro*. *Stain Technol.* **33**, 73.

ROUSE, H. C., BONIFAS, V. H. & SCHLESINGER, R. W. (1963). Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. *Virology* **20**, 357.

SANDBERG, A. A. (1966). The chromosomes and causation of human cancer and leukemia. *Cancer Res.* **26**, 2064.

SOMERSON, N. L. & COOK, M. K. (1965). Suppression of Rous sarcoma virus growth in tissue culture by *Mycoplasma orale*. *J. Bact.* **90**, 534.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. F Emb diploid cell line at 12th passage. The fibroblastic morphology of cells which have been passaged less than 30 times is clearly evident. × 170.

Fig. 2. FEL cell line at 42nd passage. These cells have lost their fibroblastic morphology and have become polygonal. × 170.

Fig. 3. F Emb cell line at 67th passage. After sixty passages these cells with a modal chromosome number of 37 (see also fig. 6) show foci of low cell density circumscribed by bands of cells stacked in a disorganized arrangement. × 170.

PLATE 2

Fig. 4. Normal chromosomes of the domestic cat from 3rd passage F Emb culture. × 1130.

Fig. 5. Karyotype prepared from fig. 4. × 1500.

Fig. 6. Karyotype prepared from 55th passage F Emb culture. × 1000.

Fig. 7. Karyotype prepared from 48th passage FEL culture. This nucleus contained 36 chromosomes. In addition to translocation on to chromosome 2 and ring formation at chromosome 6, a further translocation has resulted in the formation of a marker chromosome. × 1330.

PLATE 3

Fig. 8. FEL diploid cell line at 26th passage 40 hr. after infection with FIE virus. Three stages of intranuclear change are shown: *A*, early; *B*, more advanced and *C*, terminal at which time the cells become detached from the glass. There is also thinning of the cell sheet. × 250.

Fig. 9. F Emb cell line at 67th passage 48 hr. after infection with FIE virus. The three stages of intranuclear destruction described in fig. 8 are shown. With high passage cells, the nuclear changes following infection occur earlier and are more widespread than with low passage cells. × 250.

PLATE 4

Fig. 10. FEL diploid cell line at 25th passage 72 hr. after infection with FIE virus. There is no observable CPE in this Roux bottle which cannot be distinguished from an uninfected control bottle. × 50.

Fig. 11. FEL diploid cell line at 25th passage 72 hr. after infection with FIE virus. This is another Roux bottle inoculated simultaneously with same dose and virus used to infect the bottle in Fig. 10. In this bottle there is thinning of the cell sheet and a tendency for the cells to string out and show discrete clusters of degenerate cells.

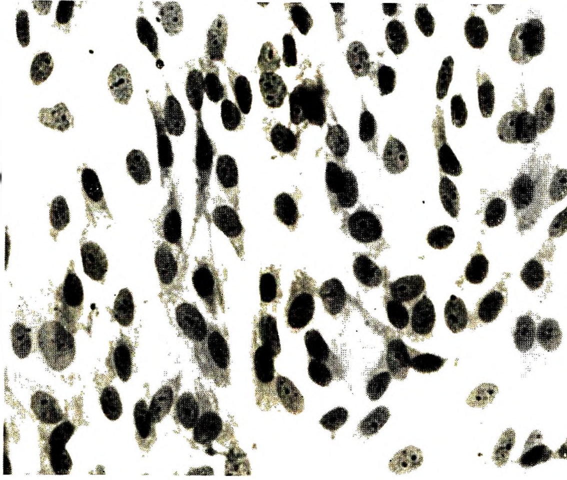


Fig. 1

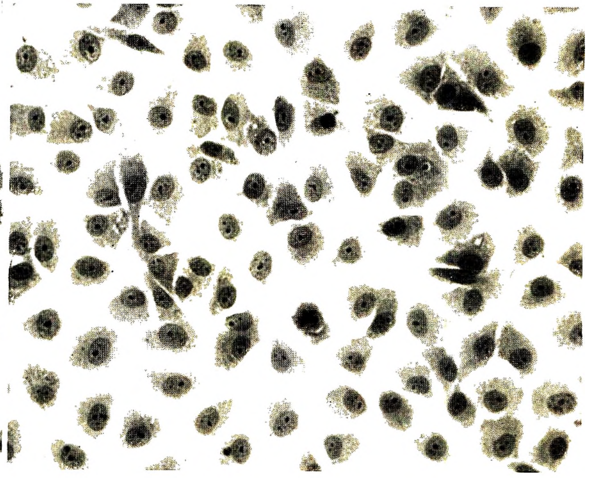


Fig. 2

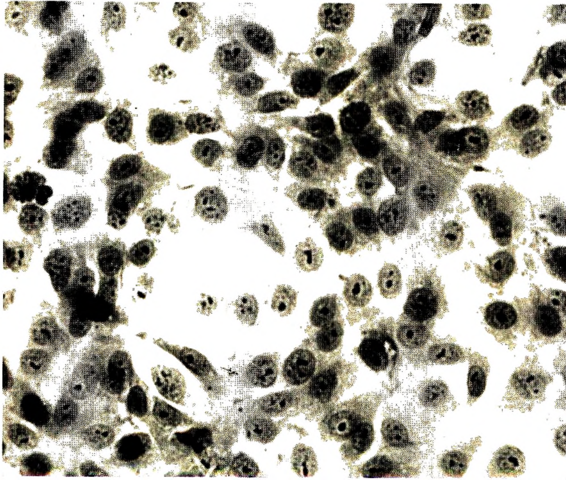


Fig. 3

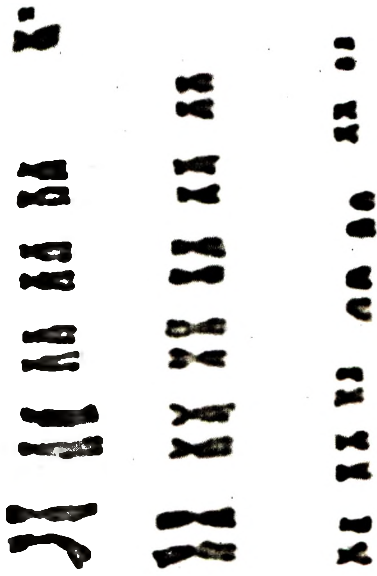


Fig. 5

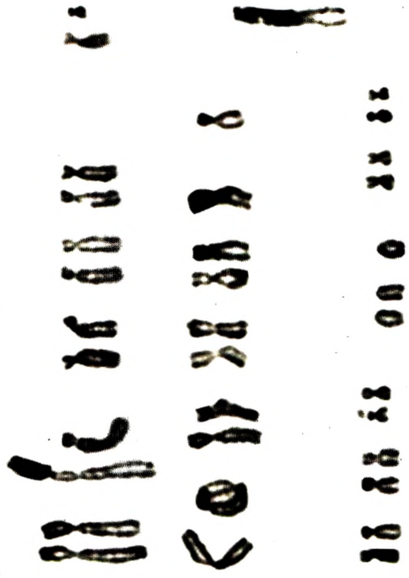


Fig. 7

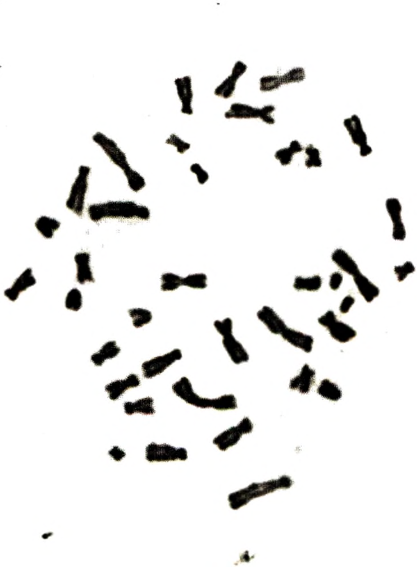


Fig. 4

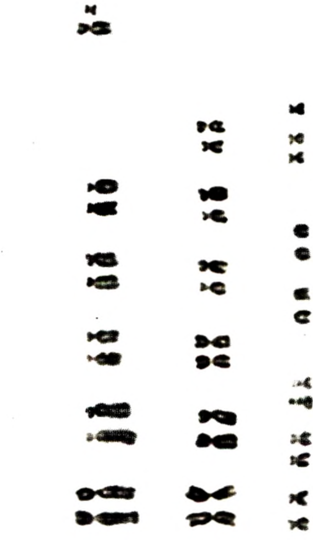


Fig. 6

K. J. O'REILLY AND A. M. WHITAKER

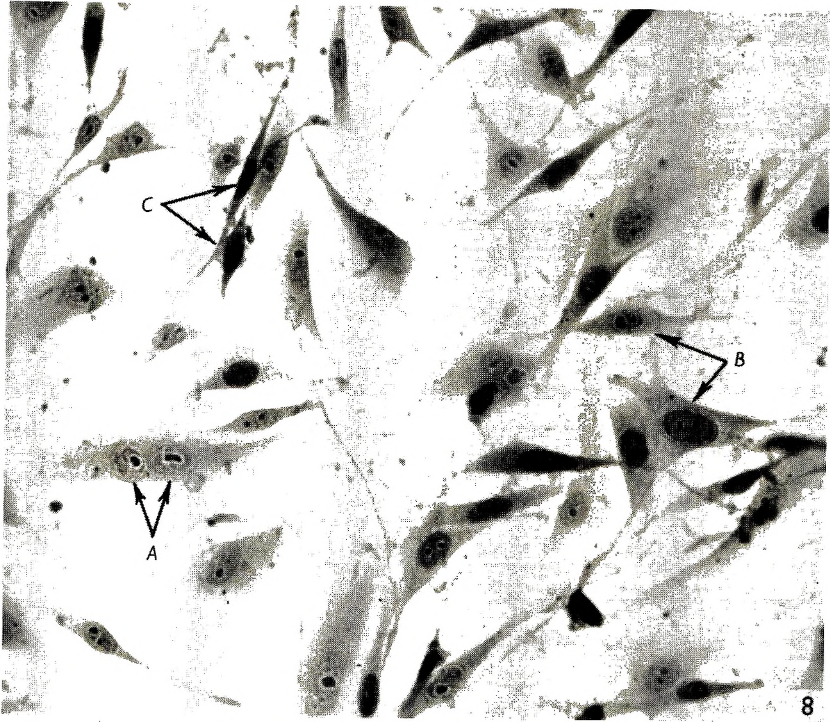


Fig. 8

Fig. 9

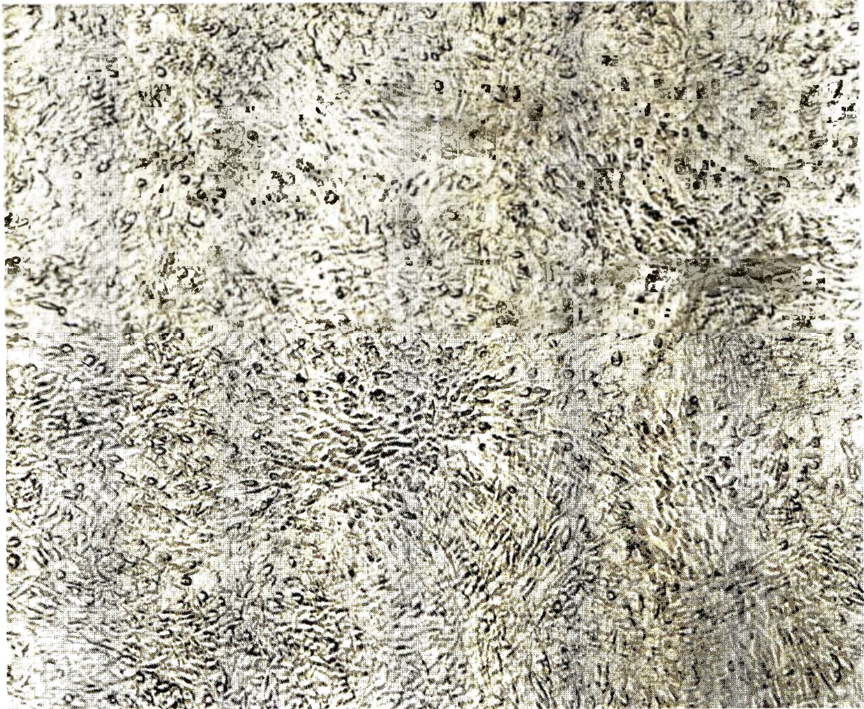


Fig. 10

Fig. 11

K. J. O'REILLY AND A. M. WHITAKER

J. Hyg., Camb. (1969), **67**, 125
Printed in Great Britain

The occurrence of *Coxiella burnetii* in North-Western England and North Wales

A report from five laboratories of the Public Health Laboratory Service*

(*Received* 27 *July* 1968)

INTRODUCTION

Marmion & Stoker (1958) discussing the epidemiology of Q fever in Great Britain conclude that raw milk is the main source of infection, whilst contact with sheep, especially in the spring during lambing and shearing time, accounts for a proportion of the cases, the placenta and wool sometimes being heavily contaminated (Stoker *et al.* 1955, Welsh *et al.* 1958). Antibody to *C. burnetii* occurs twice as commonly amongst those exposed to sheep and cattle or materials from them compared with those not so exposed (Marmion & Stoker, 1958). There is, however, no evidence that tick bites transmit the disease to man although *Haemaphysalis punctata* found on sheep and cattle may be infected (Stoker & Marmion, 1955). Henderson (1967) investigating 1052 human sera has found a complement-fixing titre of 1/8 to 1/64 or higher in 20 % of persons and is of the opinion that *C. burnetii* is of high infectivity but low pathogenicity. These and similar observations have led workers in five Public Health Laboratories in the North-West—Chester, Conway, Liverpool, Manchester and Preston—to make a retrospective survey of patients diagnosed serologically as suffering from Q fever during the 5 years 1962–6. Information has also been collected on patients during the same period whose serum titres suggested that infection had occurred at some time in the past. These results are compared with those found in persons occupationally exposed to infection, veterinary surgeons, abattoir workers and farm families, as well as with some from samples of the general population.

Since cow's milk is a known source of *C. burnetii* infection a survey has been made between 1965 and 1967 in the seven counties of Anglesey, Caernarvon, Merioneth, Flint, Denbigh, Cheshire and Lancashire of samples of milk predominantly from farms which produce milk for sale untreated (Fig. 1).

METHODS

Serological test for antibodies to Coxiella burnetii

Sera were tested for the presence of antibody to *C. burnetii* by the complement fixation test (C.F.T.) of Bradstreet & Taylor (1962) with minor modifications in the different laboratories. The antigen used routinely throughout the studies was

* The following members of the Public Health Laboratory Service took part in the investigation: J. H. Pennington, Pauline M. Poole (Chester), A. J. Kingsley Smith, K. L. Thomas (Conway), R. V. Noble, G. B. B. White, (Liverpool), A. E. Eldridge, J. O'H. Tobin, Molly W. Watkinson (Manchester), I. D. Farrell, L. Robertson (Preston). The report was prepared by Pauline M. Poole, to whom requests for reprints should be addressed at the Public Health Laboratory, Chester City Hospital, Hoole Lane, Chester.

phase 2 of the Nine Mile strain. An antigen prepared from uninfected yolk sacs was used as a control. Sera were tested in doubling dilutions starting at 1/8 or 1/10.

Screening of milk for Coxiella burnetii *infection*

Between 50 and 300 ml. of milk were centrifuged, the amount varying in the different laboratories, and the deposit resuspended in 2 ml. of gravity cream. One ml. of the mixture was inoculated intramuscularly into the thigh of a guinea-pig which was killed 6 weeks later and the serum examined for *C. burnetii* antibody by the C.F.T.

RESULTS

Patients

Sera from 7046 patients were examined for *C. burnetii* antibodies. Forty-one were considered to be suffering from recent infection at the time the samples were taken.

Q fever cases

A positive diagnosis of Q fever was made in the illness under investigation when two specimens of sera taken from a patient at intervals of generally about 10 days, but varying from 4 days to 2 years, were titrated in parallel and showed a fourfold or greater change in complement-fixing titre. A titre of 1/128 was considered to be highly suggestive of recent infection when only a single sample was available for testing or when at least one of a pair of samples attained this level, although the fourfold change was not shown. (Fourfold changes in individual patients varied from as little as $< 1/20$–1/40 to as high as 1/1280–1/5120.)

Thirty-one persons showed a significant rise in titre and were diagnosed as suffering from Q fever, and ten had titres suggestive of recent infection (Table 1). Thirty-nine were resident in the seven counties whose milk supply was investigated and their place of residence is indicated by an × on the map (Fig. 1). The total population for these counties in 1965 was 7,183,190 (Registrar General's report). Thirty-three patients were male and eight female. The number of patients varied little during each of the 5 years, but the incidence was higher in the spring, April accounting for 13 cases.

The age of 40 of the 41 patients was known, 28 (70 %) being between 20 and 60 years. Of the 7 who were less than 20 years 4 were babies in the first 2 years of life. Eleven patients gave a relevant history of possible exposure to *Coxiella.* Six were at occupational hazard. These were a butcher, a poultry specialist, a constructional engineer and a scaffolder both of whom had been concerned in the demolition of farm buildings, an excavator driver who became ill 1 week after his return from a stay in the country during which he had been present at milking and had drunk raw milk, and a wool sterilizer. Five persons in addition to the excavator driver mentioned above gave a definite or possible history of drinking raw milk. One of these, a school-teacher, invariably drank raw milk, but an extensive investigation of the herd from which the milk came was completely negative for *C. burnetii* infection.

Table 1. *Cases diagnosed as Q fever*

			Day of disease on which specimens collected		Reciprocal of titres	
Age	Sex	Diagnosis	First	Second	First	Second
19	M	Pneumonitis	9	19	< 20	160
37	M	Pneumonia	10 days*		10	1280
43	M	Pneumonia	7	12	< 5	160
?	M	Pneumonia	4	22	< 20	160
55	F	Pneumonia	8	15	< 10	80
21	M	Meningism	5	14	20	160
53	M	Pneumonia	19	44	1280	5120
56	M	Pneumonia	24	38	256	256
42	M	Meningism	2	12	< 10	640
45	M	Meningism	4	14	< 10	640
22	F	Meningism	7	17	160	640
68	M	Meningism	5	15	20	160
71	F	Pneumonia	21	34	640	640
35	M	Meningism	5	17	< 20	80
37	M	Pneumonia	6	16	20	80
73	M	Pneumonia	12	42	< 20	40
38	M	Endocarditis	212	—	128 phase 2	—
					512 phase 1	—
45	M	Pneumonia	14	80	80	320
25	F	Influenza	14 days*		20	80
73	M	Pneumonia	11	26	10	320
57	M	Influenza	35	700	640	40
45	F	Pneumonia	18	49	32	256
50	F	Pyrexia	42	52	128	1024
44	M	Pneumonia	7	14	20	640
47	F	Pneumonia	6	20	20	80
30	M	Pneumonia	7	19	20	160
4/12	M	Pneumonia	6	21	< 20	> 320
22	M	Pneumonia	3	70	16	128
28	M	Meningitis	15	24	< 8	> 128
27	M	Pneumonitis	14	21	256	256
62	M	Pneumonia	16	26	160	320
52	M	Pericarditis	4	24	640	640
7	M	Lymphadenopathy	6	37	40	160
2/12	M	Pneumonia	9	27	< 20	80
11/12	M	Lymphadenopathy	19	—	> 320	—
43	M	Pneumonia	8	15	80	> 320
1½	M	Meningism	7	20	160	160
56	M	Pneumonia	6	10	< 8	256
24	M	Meningism	1	17	8	64
23	F	Meningism	14	42	128	128
2½	M	Cerebellar ataxia	?	?	8	64

* Interval between collection of first and second specimen, the dates of which are not known.

Clinical manifestations

Pulmonary. Twenty-five patients were variously described as suffering from pneumonia, virus pneumonia, atypical pneumonia, pneumonitis or influenza, some with consolidation of the lung, with or without pleurisy. Headache was specifically mentioned in three of these 25, one of whom had photophobia and neck stiffness.

Fig. 1. Anglesey: 138 farms, 24 Q+; 247 samples, 43 Q+. Caernarvon: 148 farms, 9 Q+; 268 samples, 22 Q+. Merioneth: 123 farms, 5 Q+; 268 samples, 5 Q+. Denbigh: 27 farms, 3 Q+; 52 samples, 5 Q+. Flintshire: 52 farms, 12 Q+; 62 samples, 13 Q+. Cheshire: 232 farms, 47 Q+; 825 samples, 98 Q+. Lancashire: 1004 farms, 103 Q+; 2627 samples, 155 Q+. ×, Location of cases by residence. ●, Location of infected farms.

Two patients were jaundiced, another had weakness of the face and foot drop. The wife and daughter of one patient diagnosed serologically as suffering from Q fever pneumonia also suffered from pneumonia about the same time but the sera were not examined.

Meningeal. In nine patients the main symptoms were headache, neck stiffness and pyrexia. No mention was made of chest involvement. One patient suffered from meningitis, the cerebrospinal fluid giving a white cell count of 88 with 97 % lymphocytes; the chest was definitely stated to be clear.

Cardiac. The heart was affected in two cases. A man of 38 years became ill with endocarditis in August 1962. When his serum was examined in April 1963 the C.F.T. gave a titre of 1/128 to the phase 2 antigen. This serum was also titrated with phase 1 antigen (Stoker & Fiset, 1956) and was positive at a titre greater than 1/512. A mitral valvotomy was carried out in June 1964. The patient died in August 1965. The second patient, a man aged 54, suffered from pericarditis.

Miscellaneous. Two patients had lymphadenopathy, a third a pyrexia of 6 weeks duration, and a fourth cerebellar ataxia.

Evidence of infection at some time in the past

Patients whose sera gave a titre of at least 1/20 but less than 1/128 and did not, when paired sera were available, exhibit a fourfold change, were assumed to have been infected at some time.

Sera from 146 of 7005 patients, lacking evidence of recent infection, gave complement-fixing titres indicative of infection at some time in the past. In 79 the titre was 1/20; in 48, 1/32 or 1/40; in 17, 1/64 or 1/80; and in 2, 1/128. Although a single titre of 1/128 was the criterion for diagnosis of infection in the illness under study, two children with this titre are included in this group since the disease they were suffering from at the time was definitely proved to be other than Q fever.

Very little relevant information is available on these patients. Twenty-four had clinical syndromes resembling those found in Q fever, five had animal or agricultural contacts. Only one was known to have drunk raw milk.

Persons occupationally exposed to infection (*Table* 2)

Sera from veterinarians, abattoir workers, farm families and samples of the general population were screened by the C.F.T. at a dilution of 1/20 and those positive at this dilution were titrated.

Veterinarians

Blood was collected from 61 veterinarians, members of the Lakeland Veterinary Society and the Lancashire and Cheshire Veterinary Society. Fifty-two were in large-animal practice and their work was predominantly with cattle. Seventeen of 61 (28 %) had Q antibody at a titre of 1/20 or higher.

Abattoir workers

Serum from 29 of 87 (33 %) abattoir workers gave titres of 1/20 or higher. One aged 16 years had a titre of 1/320. He gave a history of a severe attack of 'influenza' 9 months earlier, 3 months after he began work.

Farm families

Twenty-four of 193 (13 %) males and females had *C. burnetii* antibody detectable at a dilution of 1/20 or more. There is however a difference in incidence between men and women, 11 of 118 men (9·2 %) and 13 of 75 women (17 %) showing evidence of infection.

Table 2. *Comparison of serum titres in different sections of the community*

	Numbers of sera with reciprocal C.F. titre of:						
	< 20	20	32–40	80	160	320	Total
Veterinarians	44	13	4	0	0	0	61
Abattoir workers	58	18	10	0	0	1	87
Farm families							
Males	107	8	2	0	1	0	118
Females	62	7	5	1	0	0	75
Antenatal sera							
Liverpool	158	3	0	0	0	0	161
Preston	246	1	0	0	0	0	247
Blood donor sera from Manchester	84	2	1	0	0	0	87
Sera from N. Wales	148	2	0	0	0	0	150
Sera from Chester	50	1	1	0	0	0	52

Table 3. *Number of infected farms and milk samples found in* 1965–67

	Counties							
	Anglesey	Caernarvon	Merioneth	Denbigh	Flint	Cheshire	Lancashire	Total
Farms selling untreated milk, 1965–7	94	185	157	162	174	c. 265	1530	
Farms tested	138*	148	123	27	52	232	1004	1724
Farms positive	24	9	5	3	12	47	103	203
Percentage positive	17	6	4	11	23	20	10	11·7
Milk samples tested	247	268	268	52	62	825	2627	4349
Samples positive	43	22	5	5	13	98	155	341
Percentage positive	17	8	2	10	21	12	6	7·8
			Slavin (1952)					
Farms tested	50	107	—	3	7	149	75	—
Farms positive	—	—	—	—	—	20	8	—
Percentage positive	—	—	—	—	—	13	11	—

* Farms selling treated milk have been tested in some instances.

General population (*Table* 2)

Antenatal patients

Two series of antenatal patients were studied. Of 161 sera examined at Liverpool three (1·9 %) contained antibody at a dilution of 1/20. Of 246 examined at Preston one (0·4 %) contained antibody at this level.

Blood donors

Sera from 87 blood donors living in an area where milk was known to be consumed largely unpasteurized were tested at Manchester. Two sera fixed complement at a dilution of 1/20 and one at 1/32.

Miscellaneous

Sera came variously from waterworks employees, factory workers and patients under investigation for venereal disease. In North Wales 150 sera were examined, of which two contained antibody at 1/20. In Chester, out of 52 sera, one contained antibody at 1/20 and one at 1/40.

Milk

Tests on milk samples

In all, 4349 samples of milk from 1724 farms were examined in the counties of Anglesey, Caernarvon, Merioneth, Denbigh, Flint, Cheshire and Lancashire during the period 1965–7. Three hundred and forty-one samples (7·8 %) from 203 farms (11·7 %) were found to be infected (Table 3). The number of times milk from any one farm was examined varied widely. Some farms were tested repeatedly, others on one occasion only. Where frequent sampling was carried out it was often only after several examinations that a positive result was obtained. Similarly the proportion of farms sampled varied from county to county, but was fairly extensive other than in Denbigh and Flint. The positions of infected farms are shown by a dot on the map.

The rate of infection was highest in Flintshire with 23 % of farms and 21 % of samples positive, and lowest in Merioneth with 4 % of farms and 2 % samples positive.

The possibility of natural infection of guinea-pigs with *C. burnetii* can be excluded since no antibody could be demonstrated *post mortem* in 85 guinea-pigs inoculated with specimens other than milk, and small pools of sera from guinea-pigs inoculated with specimens other than milk are used regularly as a source of complement in the C.F.T. and no evidence of antibodies to *C. burnetii* has been found. Similarly the possibility of infection being spread between guinea-pigs sharing the same cage can be excluded. Of nine pairs of cage-mates examined, in each instance only one of the pair was found to have antibody to *C. burnetii*.

DISCUSSION

Forty-one (0·57 %) cases of *C. burnetii* infection were diagnosed in 7046 patients investigated serologically for the presence of viral and rickettsial antibodies during the years 1962–6 compared with 0·65 % of 3500 patients in 1952 and 1953 (MacCallum, 1954). They showed the usual male-to-female predominance with the large majority occurring during active adult life (Clark, Lennette & Romer, 1951; Harvey, Forbes & Marmion, 1951; Stoker, 1954; Marmion & Stoker, 1958; Connolly, 1968). The incidence was highest in Spring (Clarke, Lennette & Romer, 1951; Marmion & Stoker, 1958). In just over a quarter of the patients there was evidence

of occupational hazard or a history of drinking raw milk. The remainder gave no relevant history.

A further 146 patients showed evidence of infection in the past and a proportion of these may have suffered from *C. burnetii* infection in the illness under investigation although their serological titres did not satisfy the criterion for a definite diagnosis. This may well have been so since, in the proved cases, in nine of those showing a fourfold or greater change in titre the higher titre was 1/80 or less. However, a single titre of this order cannot be unequivocally interpreted as diagnostic, since antibody may remain appreciably high for several months after the illness (Lennette *et al.* 1952). Indeed the serum of one abattoir worker screened in this survey gave a titre as high as 1/320 nine months after the probable acute illness.

The higher incidence of antibodies in those occupationally exposed to infection—veterinarians, workers in the abattoir and farm families—when compared with the general population reflected the findings of other workers (Marmion & Stoker, 1958; Connolly, 1968). In this study a higher percentage of veterinarians and workers in the abattoir showed evidence of infection than did members of farm families. This may be explained by the heavier exposure experienced by the two former groups, of which the veterinarians in particular handle the placenta, which may be grossly infected (Welsh *et al.* 1958), and workers in the abattoir are constantly exposed to the aerosols produced during killing and processing of animals. The greater incidence of antibody amongst the female members of farm families compared with the male was of special interest since clinical infection is far commoner in the male than in the female. Women on the farm usually look after the dairy and on producer–retailer farms are largely responsible for cleaning, filling and capping milk bottles and therefore have more contact with raw milk than the men, who do the actual milking but with modern machinery. This may result in constant exposure to small amounts of antigen and hence the development of antibody without manifestation of clinical disease. Stoker (1954) found that, in sera from healthy blood donors, the proportion of males to females with antibody was much less than the numbers with clinical disease would lead one to expect. He postulated that, perhaps owing to smaller dosages or to constitutional differences, women were less susceptible to clinical illness. In a short sharp outbreak described by Harvey, Forbes & Marmion (1951), despite an equal degree of exposure the proportions of males to females with clinical illness was 20 to 7. Whether the apparent increased incidence in blood donors tested at Manchester was due to drinking unpasteurized milk or other type of exposure is difficult to determine.

A higher rate of infection of milk was found in the present study when compared with that of Slavin (1952) except in Lancashire, where the percentage of infected farms was similar. He, however, used Henzerling antigen and sampling was on a smaller scale. In this study some farms were investigated only infrequently and more intense sampling might have revealed an even higher incidence of infection.

Despite the wide distribution of *C. burnetii* in milk and the heavy exposure to infection of some groups of the community, the fact remains that sporadic overt Q fever is not as commonly diagnosed as one might expect. This may be accounted

for by low pathogenicity of the organism, giving rise to subclinical infection only, by the under-use of diagnostic facilities, or by the mode of entry of the rickettsia to the body being sub-optimal for the establishment of disease.

SUMMARY

A retrospective survey of *Coxiella burnetii* infection in North-Western England and North Wales was made in five Public Health Laboratories for the years 1962–6 inclusive. Groups of the normal population and of persons occupationally exposed to infection were tested for the presence of antibody and the incidence of infected milk was investigated in seven counties.

Forty-one cases of *C. burnetii* infection were diagnosed in 7046 patients investigated serologically for the presence of viral and rickettsial antibodies. In just over a quarter of the patients there was evidence of occupational hazard or a history of drinking raw milk. The remainder gave no relevant history.

We would like to express our thanks to Dr C. M. Patricia Bradstreet of the Standards Laboratory, Colindale, for the supply of antigen, and to the numerous clinicians who allowed us access to their case records.

REFERENCES

Bradstreet, C. M. P. & Taylor, C. E. D. (1962). Technique of complement-fixation test applicable to the diagnosis of virus diseases. *Mon. Bull. Minist. Hlth* **21**, 96.

Clark, W. H., Lennette, E. H. & Romer, M. S. (1951). Q fever in California. XI. An epidemiologic summary 1948–1949. *Am. J. Hyg.* **54**, 319.

Connolly, J. H. (1968). Q fever in Northern Ireland. *Br. med. J.* i, 547.

Harvey, M. S., Forbes, G. B. & Marmion, B. P. (1951). An outbreak of Q fever in Kent. *Lancet* ii, 1152.

Henderson, R. J. (1967). Brucellosis in the dairy farming community and allied workers of Worcestershire. *Lancet* ii, 353.

Lennette, E. H., Clark, W. H., Jensen, F. W. & Toomb, C. J. (1952). Development and persistence in man of complement fixing and agglutinating antibodies to *Coxiella burnetii*. *J. Immun.* **68**, 591.

MacCallum, F. O. (1954). Discussion following previous papers. *Trans. R. Soc. trop. Med. Hyg.* **48**, 203.

Marmion, B. P. & Stoker, M. G. P. (1958). Epidemiology of Q fever in Great Britain. *Br. med. J.* ii, 809.

Slavin, G. (1952). 'Q' fever: the domestic animal as a source of infection for man. *Vet. Rec.* **64**, 743.

Stoker, M. G. P. (1954). Q fever. I. Clinical features and laboratory diagnosis. *Trans. R. Soc. trop. Med. Hyg.* **48**, 191.

Stoker, M. G. P., Brown, R. D., Kett, F. J. L., Collings, P. C. & Marmion, B. P. (1955). Q fever in Britain: isolation of *Rickettsia burneti* from placenta and wool of sheep in an endemic area. *J. Hyg., Camb.* **53**, 313.

Stoker, M. G. P. & Fiset, P. (1956). Phase variation of the nine mile and other strains of *Rickettsia burneti*. *Can. J. Microbiol.* **2**, 310.

Stoker, M. G. P. & Marmion, B. P. (1955). Q fever in Britain: isolation of *Rickettsia burneti* from the tick *Haemaphysalis punctata*. *J. Hyg., Camb.* **53**, 322.

Welsh, H. H., Lennette, E. H., Abinanti, F. R. & Winn, J. F. (1958). Airborne transmission of Q fever: the role of parturition in the generation of infective aerosols. *Ann. N.Y. Acad. Sci.* **70**, 528.

J. Hyg., Camb. (1969), **67**, 135
Printed in Great Britain

Salmonella and Arizona in reptiles and man in Western Australia

BY J. B. IVESON, E. M. MACKAY-SCOLLAY AND V. BAMFORD
Salmonella Diagnostic and Reference Laboratory, Public Health Laboratory Service, Western Australia

(*Received* 30 *July* 1968)

INTRODUCTION

During the course of investigations into the prevalence of *Salmonella* in food-stuffs produced in Western Australia, and as a result of findings in routine medical diagnostic bacteriology, it was evident that a wide variety of serotypes existed in the State. During a 5-year period seventy distinct serotypes were isolated from human infections in the community. Further, it was remarked that a geographical distribution of serotypes existed. A relative prominence of salmonellas with numerically high somatic antigens, the isolation of new serotypes, the incidence of multiple infections, and the finding of strains in *Salmonella* subgenera II, III and IV were associated with specimens which originated in the remoter and more recently developed areas of the north. The converse was found in the metropolitan district of Perth and the more populous areas of the south-west.

A possible explanation for this geographical distribution of serotypes was the ecological relationship of *Salmonella* species and the fauna of the country. It appeared that in the remoter regions the strains were originating in a reservoir of infection that was unlikely to be human owing to the sparseness of the population and the diversity of types isolated from human cases of salmonellosis. Since the geological history of the Australian subcontinent is one of evolutionary development in relative isolation from the land masses of the world, a fact illustrated in the unique fauna of reptiles, marsupials and the last surviving monotremes, and, since isolations of salmonellas were made from reptiles caught in areas remote from human habitation, it was reasoned that these creatures might serve as the reservoir of types that had evolved independently of strains in other parts of the world.

A limited investigation was undertaken to assess the importance of reptiles in Australia as carriers of *Salmonella* and Arizona groups of organisms and this report records the findings of our study.

MATERIALS AND METHODS

Specimens

One hundred and sixteen reptiles, comprising 70 lizards, 40 snakes, 4 tortoises and 2 crocodiles, originating in Australia were examined. At least five of the snake species examined were venomous. The size of the reptiles ranged from a 14 ft. python to legless lizards a few inches long. Specimens were obtained from captive creatures and from those in their natural environment either remote from or near

to human habitation. Rectal swabs from a number of native birds and rodents, from a domestic cat and a captive kangaroo, all of which were living in close association with some of the reptiles, were examined. In addition, sixteen samples of litter from captive reptiles were tested; this litter was contaminated by rodent litter from live animals fed to snakes. Some of the larger lizards were infested with ticks which were removed, macerated and cultured separately.

The majority of specimens were taken on a single examination, but repeat specimens were taken from two wild lizards after 6 weeks in captivity, and on several occasions from two captive pythons; the last occasion being 9 months after first sampling and following a period of hibernation. Samples of contaminated soil and reptile litter, stored in sealed jars, were examined after an interval of 12 weeks from first examination. This repeat sampling was a part of survival experiments to be reported elsewhere.

Cloacal contents on swabs were placed in 1–5 ml. volumes of Sachs's (1939) buffered glycerol saline transport medium. Organs from reptiles submitted dead or diseased were removed with aseptic precations. All samples were homogenized before processing.

Culture procedure

All samples were first inoculated direct on SS agar (Oxoid or Difco) and modified bismuth sulphite agar (BSA) (Hobbs, 1943). Approximately 0·5 ml. of each sample, delivered by pasteur pipette, was sown into 10 ml. volumes of enrichment media of Leifson (1936), and of Rappaport, Konforti & Navon (1956) (as modified by Iveson & Kovacs, 1967), and of selenite F. broth. Also included were two new enrichment media (to be published separately) designed to recover a wider range of salmonella serotypes from human and animal sources and from food. Subcultures were made from all enrichment media after 18–24 hr. incubation, and occasionally after 48 hr, at 37° C. on SS and modified BSA media.

The BSA medium was prepared by reconstituting dehydrated bismuth sulphite agar (Oxoid or Difco) as recommended by the manufacturer, but, immediately before pouring into plastic Petri plates, 10 ml. of 1 % ferrous sulphate and 3·0 ml. 10 % ferric citrate were added to 1 litre of the molten medium. Plates were dried for 30–45 min. with the lids removed, and were stored at 4° C. overnight or up to 4 days before use. The additive solutions were sterilized by heating at 60° C. for 1 hr., and remained stable for several weeks at 4° C.

Non-lactose fermenting colonies on SS agar, or colonies resembling *Salmonella* or Arizona species on the modified BSA medium were examined biochemically by inoculation on a composite medium slope, to differentiate subgenus I and III strains and related Enterobacteriaceae. The differential composite medium detected fermentation of glucose, lactose, sucrose, sorbose, mannitol, dulcitol, production of hydrogen sulphide and splitting of urea after 16–24 hr. incubation. Growth from the slope was used for serological testing and further biochemical reactions. Dulcitol-negative cultures were further screened biochemically for reactions with dulcitol and sorbose peptone water, lysine, malonate, gelatin, and O.N.P.G. tests. Serological confirmation of subgenus III strains was made where

appropriate. The use of sorbose, as reported by Kauffmann (1956) and Stenzel (1960), coupled with lysine reaction served to differentiate *Citrobacter* spp. Colony selection was limited to a maximum of five colonies per plate, or an average of fifteen for each specimen. During the investigation up to fifty colonies were serologically screened from selected specimens as a rough check on routine recoveries.

RESULTS

From 116 reptiles examined 97 yielded *Salmonella* or Arizona species (Table 1). Of 70 lizards in the investigation 54 (77 %) were positive and among 40 snakes tested 37 (92 %) proved positive. The 4 tortoises and 2 crocodiles examined were all positive for *Salmonella* but negative for Arizona. Forty-four (63 %) lizards yielded *Salmonella* only (5 infected with more than one strain) and 4 (6 %) were positive for Arizona only; whereas 4 (10 %) snakes were positive for *Salmonella* only, while 15 (37 %) were positive for Arizona only. The distribution of serotypes was: 29 salmonella and 7 Arizona serotypes in lizards, compared with 14 salmonella and 10 Arizona serotypes in snakes.

Table 1. *Isolations of salmonella and Arizona strains from* 116 *reptiles*

Reptile	No. tested	No. positive	No. positive for: Salmonella only	No. positive for: Arizona only	No. of: Salmonella serotypes	No. of: Arizona serotypes
Lizard	70	54 (77)	44 (63)	4 (6)	29	7
Snake	40	37 (92)	4 (10)	15 (37)	14	10
Tortoise	4	4	4	0	4	0
Crocodile	2	2	2	0	2	0
Totals	116	97 (84)	54 (47)	19 (16)	49	17

Figures in parentheses indicate percentages

The distribution of salmonella and Arizona serotypes in the ninety-seven reptilian genera and species from which positive isolations were made is shown in Table 2. In general, the reptiles imported from Australian states, and those in the southern region of West Australia (including metropolitan Perth), were captive creatures or were caught in proximity to human habitation. Those reptilia caught in the northern region of the state (which has been demarcated for convenience from the southern region at 28° latitude) were free-ranging. There appeared to be no peculiar distribution of serotypes as between captive and wild reptiles, although *S. typhimurium* was absent from specimens taken in geographically remote regions, whether from wild or captive reptiles; neither was *S. typhimurium* isolated from the birds, domestic cat or rodents which were sampled in the north of the state although five isolations from the birds and the domestic cat were made. However, *S. typhimurium* was occasionally found in reptiles, including snakes, living in captivity in the metropolitan area. *S. typhimurium* was isolated from rodents fed to these snakes and from the mixed rodent and reptile litter.

From ten litter samples associated with the reptiles imported from the Eastern

Table 2(*a*). *Salmonella and Arizona isolations from fifty-four Australian lizards*

Lizards			*Salmonella*			*Arizona*			
	State of origin	No.	Serotype	No.	Total no.	Serotype	No.	Total no.	Total isolations
Amphibolurus barbatus	NSW	1	*ohlstedt*	1	1	.	.	.	1
	Vic	2	42:Z; –	1	1	5:29-21	1	1	2
	WA 1	1	*bullbay*	1	.	.	.	.	.
			nashua	1	2	.	.	.	2
	WA 2	8	*adelaide*	2	.	.	.	.	.
			boecker	1	.	.	.	.	.
			chester	1	.	.	.	.	.
			emmastad	1	.	.	.	.	.
			kisarawe	6	.	.	.	.	.
			*mowanjum**	1	.	.	.	.	.
			muenchen	1	.	.	.	.	.
			orion	2	.	.	.	.	.
			rubislaw	1	.	.	.	.	.
			senftenberg	4	.	.	.	.	.
			tenessee	1	.	.	.	.	.
			wandsworth	1	22	.	.	.	22
A. ornatus	WA 1	11	*kisarawe*	4	.	.	.	.	.
			nashua	9	13	.	.	.	13
Diporiphora bilineata	WA 2	1	47:k:–	1	1	.	.	.	1
Lialis burtoni	WA 1	2	*hvittingfoss*	1	.	.	.	.	.
			newport	1	2	.	.	.	2
Moloch horridus	WA 1	1	*chester*	1	1	.	.	.	1
Omolepida branchiale	WA 1	1	*muenchen*	1	1	.	.	.	1
Physignathus leseurii	Q	4	*chester*	2	.	1,33:23–21	1	.	.
			adelaide	1	.	28:23–25	1	.	.
			rubislaw	1	.	29:24–31	1	.	.
			wandsbek	2	6	.	.	3	9
Tiliqua occipitalis	WA 1	2	*alsterdorf*	1	.	.	.	.	.
			lindern	1	2	.	.	.	2
T. occipitalis multifasciata	WA 2	1	47:k; –	1	1	.	.	.	1
T. rugosa	Vic	1	*charity*	1	.	.	.	.	.
			give	1	2	.	.	.	2
T. scincoides	Vic	2	*chester*	1	.	.	.	.	.
			saintpaul	1	2	5:29–30	1	1	3
	WA 2	1	*adelaide*	1	.	.	.	.	.
			muenchen	1	.	.	.	.	.
			orion	1	.	.	.	.	.
			senftenberg	1	4	.	.	.	4
Trachysaurus rugosus	WA 1	3	*give*	1	.	.	.	.	.
			ohlstedt	1	.	.	.	.	.
			orientalis	1	.	.	.	.	.
			singapore	1	4	.	.	.	4
Varanus tristis	WA 1	7	*alsterdorf*	1	.	26:26–25	1	.	.
			champaign	5	.	.	.	.	.
			give	2	.	.	.	.	.
			wandsbek	1	9	.	.	1	10
V. varius	NSW	1	.	.	.	1,33:23–21	1	1	1
	Q	1	.	.	.	16:23–25	1	1	1
	SA	1	*saintpaul*	1	.	species	1	.	.
			chester	1	2	.	.	1	3
	Vic	1	.	.	.	26:24–25	1	1	1
Unidentified	WA 2	1	*kisarawe*	1	1	.	.	.	1
Total lizards		54	.	.	77	.	.	10	87

* Indicates new serotypes

Table 2(*b*). *Salmonella and Arizona isolations from thirty-seven Australian snakes*

Snakes			*Salmonella*			*Arizona*			
	State of origin	No.	Serotype	No.	Total no.	Serotype	No.	Total no.	Total isolations
Ahaetulla punctulata	Q	7	*adelaide*	1	.	1,33:23–21	1	.	.
			muenchen	2	.	15:24–31	1	.	.
				.	.	9a9b:26–21	4	.	.
				.	.	24:26–21*	1	.	.
				.	.	25:23–25*	2	.	.
				.	3	28:32–28	1	10	13
Aspidites melanocephalus	WA 2	1	*chester*	1	1	5:29–30	1	1	2
Demansia affinis	WA 1	2	*potsdam*	1	1	16:23–25	1	.	.
						Species	1	2	3
D. nuchalis	WA 1	1	.	.	.	28:32–35	1	1	1
D. olivacea	Vic	1	*newington*	1	1	1,33:23–21	1	1	2
Denisonia superba	Vic	1	*hvittingfoss*	1	.	5:29–31	1	.	.
			orientalis	1	2	.	.	1	3
Liasis amethystinus	Q	1	.	.	.	20:29–25	1	.	.
						30:22–31	1	2	2
L. childreni	Q	1	*typhimurium*	1	1	25:23–25*	1	1	2
Morelia spilotes spilotes	Q	4	*saintpaul*	2	.	16:23–25	1	.	.
			typhimurium	1	.	16:23–37	1	.	.
				.	3	30:26–21	1	3	6
M. spilotes variegata	Q	5	*chester*	1	.	16:23–25	1	.	.
			enteritidis	1	.	30:26–21*	4	.	.
			typhimurium	3	.	29:29–25*	1	.	.
				.	5	30:22–31	1	7	12
M. variegata	WA 1	1	.	.	.	29:29–25*	1	1	1
Notechis scutatus	Vic	4	*onderstepoort*	1	.	26:33–31	3	.	.
			orientalis	1	.	5:29–21	1	.	.
			typhimurium	1	.	16:27–25	1	.	.
			wandsbek	1	4	.	.	5	9
	WA 1	3	*kibusi*	1	.	16:23–25	2	.	.
			newport	1	.	.	.	.	.
			typhimurium	1	3	.	.	2	5
Pseudechis	NSW	2	.	.	.	16:23–25	1	.	.
			.	.	.	26:33–31	1	2	2
	Q	1	.	.	.	20:29–25	1	1	1
Unidentified	WA 1	2	*muenchen*	2	2	26:23–21	1	1	3
Total snakes		37	.	.	26	.	.	41	67

* Indicates new serotypes.

Table 2(*c*). *Salmonella and Arizona isolations from four Australian tortoises and two Australian crocodiles*

Tortoises and crocodiles			*Salmonella*			*Arizona*			
	State of origin	No.	Serotype	No.	Total No.	Serotype	No.	Total no.	Total isolations
Chelodina rugosa	WA 2	3	*champaign*	1	.	.	1	1	1
			orion	1	.	.	.	.	.
			emmastad	1	.	.	.	.	.
			wandsbek	1	4	.	.	.	4
Emydura australis	WA 2	1	*champaign*	1	.	.	.	.	.
			orion	1	.	.	.	.	.
			wandsbek	1	3	.	.	.	3
Crocodylus porosus	WA 2	2	*adelaide*	1	.	.	.	.	.
			O group G	1	2	.	.	.	2
Total tortoises and crocodiles		6	.	.	9	.	.	.	9

States of Australia detailed in Table 3, twenty-two isolations of salmonella and Arizona serotypes were made; while from six litter samples of reptiles and rodents in metropolitan Western Australia twelve isolations of salmonella and Arizona strains were achieved. *S. typhimurium* was isolated from both sources.

Infections by more than one serotype were frequent. Twelve multiple infections, involving three serotypes, were detected in individual lizards and snakes. One tortoise yielded three salmonella serotypes.

Table 3. Salmonella *and Arizona serotypes isolated from reptilian and associated rodent litter of captive reptiles*

Source of litter	Salmonella Serotypes	No.	Arizona Serotypes	Arizona No.	Total
Imported reptiles from eastern states (10 samples)	*adelaide* (2) *alsterdorf* (1) *blukwa* (1) *charity* (1) *chester* (3) *saintpaul* (1) *typhimurium* (2) *uzuramo* (1) *wandsbek* (6)	18	5:29–21 (2) 26:27–25 (1) 26:33–31 (1)	4	22
West Australian (6 samples)	*chester* (2) *enteritidis* (4) *saintpaul* (2) *typhimurium* (2)	10	16:23–25 (1) 16:23–27 (1)	2	12
Totals		28		6	34

The distribution of serotype recoveries in the various organs from seven dissected reptiles are shown in Table 4. Up to five serotypes were recovered from various sites in lizards, while both salmonella and Arizona types were found widely distributed in the organs of snakes, including the ovaries, stomach and gall bladder.

Of the nineteen identified serotypes of Arizona isolated from snakes only two (1,33:23–21 and 5:29–21) were also found among the seven identified strains from lizards.

DISCUSSION

Reptiles were first investigated bacteriologically during coliform studies by Bettencourt & Borges (1908), and Konrich (1910). Salmonella serotypes from lizards, snakes, and Galapagos turtles were reported by McNeil & Hinshaw (1944, 1946) and Hinshaw & McNeil (1945, 1947), who speculated that lizard infection was food-borne from human carriers and that lizards and snakes were reservoirs of infection for turkeys and chickens. Also in America, Parker & Steinhaus (1943) reported *Salmonella* infection in ticks.

Rewell, Taylor & Douglas (1948) isolated a salmonella strain from a captive West African python in England, and Boycott, Taylor & Douglas (1953), linked possible human infection with isolations from imported Moroccan tortoises describing the finding as 'both novel and alarming'. Recent examination of

Table 4. Salmonella *and Arizona serotype colony distribution in seven reptiles*

Reptile	Origin	Serotype organ distribution			Total colonies sero-typed	Sero-types
		Stomach	Other organs	Cloaca and intestine		
Notechis scutatus	Southern West Australia	Arizona 16:23–25	*S. kibusi* Arizona 16:23–25	*S. kibusi* Arizona 16:23–25	> 20	2
Amphilbolurus barbatus (3 reptiles)	Northern West Australia	*S. senftenberg* (2) *S. kisarawe* (1) *S. rubislaw* (1) *S. chester* (9) *S. adelaide* (2)	*S. senftenberg* (5) *S. wandsworth* (9) (liver)	*S. senftenberg* (9) *S. kisarawe* (13) *S. rubislaw* (3) *S. chester* (8) *S. adelaide* (5) *S. tennessee* (1) *S. orion* (1)	69	8
Tiliqua scincoides	Northern West Australia	*S. senftenberg* (9) *S. orion* (3)		*S. senftenberg* (6) *S. adelaide* (12) *S. muenchen* (9) *S. orion* (2)	41	4
Tiliqua scincoides multi-fasciata	—	*Salmonella* 47 k: – (8)	.	*Salmonella* 47 k: – (27)	35	1
Notechis scutatus	Victoria	*S. onderstepoort* *S. typhimurium* Arizona 16:27–25	Arizona 16:27–25 Arizona 5:29–21 *S. onderstepoort*	Arizona 16:27–25 . .	> 50	4

reptiles in the zoological gardens of Basle, Berne and Zurich by Rudat *et al.* (1966) provided fifteen serotypes, including three occurring in Western Australia. The authors comment that infected reptiles were of little consequence in infection trends in the attendants. The frequent presence of *Salmonella* in African reptiles has been recorded by Mackey (1955) and Collard & Sen (1960). Mackey considered the numerous house lizards observed might be primary reservoirs of human infection. He showed that among thirty-three different serotypes found in lizard droppings, twenty-one of the types were also isolated from human cases in the same area, but only one of these isolations was *S. typhimurium*. Fulton, Szafran & Lesko (1961) isolated *Salmonella* from reptiles in the Congo in an area where infection from man and rodents was unlikely and suggested that the strains obtained were present in reptiles as intestinal commensals.

In Australia, salmonella isolations from native animals were described by Lee & Mackerras (1955), who considered that the carrier state was the more usual condition in reptiles, but that adverse environmental factors might lead to disease in the host. These workers also noted the relative absence of *S. typhimurium*.

The first reported isolations of Arizona organisms from lizards were made in America by Caldwell & Ryerson (1939). Further isolations were described by Edwards, Cherry & Bruner (1943) from reptiles, fowls, mammals and man. Le Minor, Fife & Edwards (1958) isolated strains of Arizona from 134 venomous snakes obtained in France for the garnering of venom. Brookes & Fife Asbury (1966), investigating cultures from South Africa and the London Zoo, found fourteen new Arizona serotypes from snakes, a tortoise, a lizard, and from a sample of human faeces. In a total of 229 infections reported by Edwards, Fife & Ramsey (1959) 205 were accompanied by symptoms; and Krag & Shean (1959) have reported two fatal infections. Caldwell & Ryerson (1939) considered the aetiological role of Arizona in disease difficult owing to a lack of specific knowledge of reptilian disease.

Outbreaks of infection associated with subgenus III strains have been reported in France by Buttiaux & Kesteloot (1948), and in America by Murphy & Morris (1950).

The first Arizona isolation in Western Australia was made in 1962 from an infected pig gland, and 2 years later a second strain was isolated from the faeces of an 8-year old boy with acute gastro-enteritis. A further eight human isolations have been obtained from the faeces of patients between the age of 3 months and 65 years during 1967 to May 1968. All patients had gastro-intestinal symptoms; at least one continued to excrete the organism for several weeks, during which time the symptoms persisted. All the human and domestic animal Arizona isolations, including those from kangaroo and rabbit meat, were made from specimens received from remote areas, mainly in the northern regions of the State. A further subgenus IV strain was isolated from a sewage sample collected in the extreme northern Kimberley region of the State.

The absence of *S. typhimurium*, together with increased isolations of new serotypes, subgenus II, III and IV strains, as well as multiple serotype infection and re-infection in the less developed regions of the State is illustrated in Table 5, which

shows the distribution of Salmonella serotypes in human infections by the geographical regions of Western Australia. The table also shows the *S. typhimurium* distribution. Edwardsiella species, as reported by Ewing *et al.* (1965), have not yet been isolated, although we were aware of their possible presence in the later stages of the investigation.

Table 5. *Geographic distribution of* Salmonella *serotypes isolated from human faeces*

Region	Total persons	Salmonella serotypes: Subgenus I	II	III	O groups A–G	Other	*typhimurium*	Persons with multiple serotype isolations
Northern	642	616	21	5	446	191	33	68
Southern	776	773	1	2	723	51	434	15
Totals	1418	1389	22	7	1169	242	467	83

Our investigation has shown no distribution differences in the serotypes isolated from captive and free-ranging reptiles with the exception of *S. typhimurium*, which was not isolated from wild reptiles but only from captive snakes and the rodents provided for their food and their combined litter. There was a higher incidence of *Salmonella* infection in lizards than in snakes, while the reverse was found for Arizona infection.

The significance of salmonella and Arizona organisms in reptiles is complicated by our lack of knowledge of other host–parasite relationships that may be present. The ectoparasite *Amblyoma triguttatum* from lizards, included in this report, frequently contained *Salmonella* species. One lizard captured in a metropolitan cemetery yielded both *S. give* and *S. ohlstedt* from the cloaca, *S. ohlstedt* from ticks deep inside the left ear cavity, and *S. houten* from ticks in the right ear. Arizona isolations were also recorded from ticks on the larger monitor lizards (*Varanus varius*). Endoparasites and ectoparasites including intestinal flagellates, ciliates, amoebae, and haematozoa in Australian reptiles, monotremes, and marsupials have been recorded by Johnston (1932) and Mackerras (1961, 1962), the latter commenting that in the ancient group of reptiles it could be expected that their parasites would be widely distributed. However, some lizards have restricted ranges, which could lead to a relatively isolated development not only of particular species, or even races of parasite, but of particular strains of *Salmonella* or Arizona. Ten strains of *S. kisarawe*, for instance, were isolated from twenty *Amphibolura* but from only one other lizard (which was not identified).

Lizards and snakes occur throughout Australia. In Western Australia their distribution is widespread and varies from remote uninhabited desert to areas close to human habitation. This report has shown that both groups of reptiles are frequently colonized or infected with salmonella and Arizona groups of organisms. Furthermore, the evidence here presented suggests that these organisms were established in Australian reptiles before the invasion of the subcontinent by European man and his introduced fauna. Reptiles thus provide a reservoir of strains from which man

and his domestic animals can be infected or from which his food can become contaminated. The implication of this ecological relationship may extend beyond the shores of the Commonwealth to any country where reptiles abound and foods favouring the survival of enteropathogenic bacteria are produced for home or foreign consumption.

SUMMARY

1. Ninety-seven (83·6 %) of 116 reptiles, comprising 70 lizards, 40 snakes, 4 tortoises and 2 crocodiles, yielded isolations of organisms in the Salmonella and/or Arizona groups.

2. The reptiles were captive or free-ranging; the former were drawn from all states of mainland Australia, while the latter were from West Australia only.

3. The relative prominence of *Salmonella* serotypes containing numerically high somatic antigens, the finding of new serotypes, of multiple infections, and of strains in subgenera II and III was remarked.

4. The lack of evidence of differences in the serotypes isolated from captive or wild reptiles (except for the isolation of *S. typhimurium* in creatures closely associated with man and his domestic fauna), and the apparent absence of a specific geographical distribution of serotypes in reptiles, lent support to the conclusion that reptiles provide a natural reservoir for *Salmonella* and Arizona strains in Australia. The possible spill-over to man, his domestic animals and his foodstuffs is discussed.

It is a pleasure to record our indebtedness to Dr Joan Taylor for her continuous interest and support in providing confirmation and identification of many *Salmonella* serotypes; to Dr W. H. Ewing and later Dr R. Rhode for serotyping the Arizona strains; to Dr G. M. Storr, Curator of Reptiles in the Museum of Western Australia, for identifying the reptiles and to Dr W. S. Davidson, Commissioner of Public Health, Western Australia, for permission to publish.

REFERENCES

Bettencourt, A. & Borges, I. (1908). Peut-on distinguer le Coli bacille de l'homme de celui des animaux au moyen de la fixation du complement? *Archos R. Inst. bact. Câmara Pestana* Tome ii, 2, 245.

Boycott, J. A., Taylor, J. & Douglas, S. H. (1953). Salmonella in tortoises. *J. Path. Bact.* **65**, 401.

Brookes, M. M. & Fife Asbury, M. A. (1966). Twenty-two new Arizona serotypes isolated from coconut, bonemeal, dried egg, and reptiles. *Mon. Bull. Minist. Hlth* **25**, 230.

Buttiaux, R. & Kesteloot, A. (1948). Les 'B. para-coli' du groupe Arizona. Leur pouvoir pathogène chez l'homme *Annls Inst. Pasteur, Paris* **75**, 379.

Caldwell, M. E. & Ryerson, D. L. (1939). Salmonellosis in certain reptiles. *J. infect. Dis.* **65**, 242.

Collard, P. & Sen, R. (1960). Serotypes of salmonella at Ibadan, Nigeria, with special note of the new serotypes isolated in Nigeria. *J. infect. Dis.* **106**, 270.

Edwards, P. R., Cherry, W. B. & Bruner, D. W. (1943). Further studies on coliform bacteria serologically related to the genus *Salmonella*. *J. infect. Dis.* **73**, 229.

Edwards, P. R., Fife, M. A. & Ramsey, C. H. (1959). Studies on the Arizona group of Enterobacteriaceae. *Bact. Rev.* **23**, 155.

EWING, W. H., MCWHORTER, A. C., ESCOBAR, M. R. & LUBIN, A. H. (1965). *Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*. *Int. Bull. bact. Nomencl. Taxon.* **15**, 33.

FULTON, MC., SZAFRAN, P. & LESKO, N. M. (1961). Five less common *Salmonella* serotypes from Congo reptiles. *Nature, Lond.* **189**, 240.

GUCKIAN, J. C., BYERS, E. H. & PERRY, J. E. (1967). *Arizona* infection of man. *Archs intern. Med.* **119**, 170.

HINSHAW, W. R. & MCNEIL, E. (1945). Salmonella types isolated from snakes. *Am. J. vet. Res.* **6**, 246.

HINSHAW, W. R. & MCNEIL, E. (1947). Lizards as carriers of salmonella and paracolon bacteria. *J. Bact.* **53**, 715.

HOBBS, B. C. (1943). A note on the preparation of bismuth sulphite agar. *Mon. Bull. Minist. Hlth* **2**, 28.

IVESON, J. B. & KOVACS, N. (1967). A comparative trial of Rappaport enrichment for the isolation of Salmonellae from faeces. *J. clin. Path.* **20**, 290.

JOHNSTON, T. H. (1932). The parasites of the 'stumpy-tail' lizard *Trachysaurus rugosus*. *Trans. R. Soc. S. Aust.* **56**, 62.

KAUFFMANN, F. (1956). On biochemical investigations of enterobacteriaceae. *Acta path. microbiol. scand.* **39**, 85.

KONRICH. (1910). Zur Bewertung des Bacterium coli im Wasser. *Klin. Jb.* **23**, 1.

KRAG, D. & SHEAN, D. B. (1959). Serious human infection due to bacilli of the Arizona group. *Calif. Med.* **90**, 230.

LEE, P. E. & MACKERRAS, I. M. (1955). Salmonella infections of Australian native animals. *Aust. J. exp. Biol. med. Sci.* **33**, 117.

LEIFSON, E. (1936). A new selenite medium for the isolation of Salmonella. *Am. J. Hyg.* **24**, 423.

LE MINOR, L., FIFE, M. A. & EDWARDS, P. R. (1958). Recherches sur les *Salmonella* et *Arizona* hébergées par les vipères de France. *Annls. Inst. Pasteur, Paris* **95**, 326.

MCNEIL, E. & HINSHAW, W. R. (1944). Snakes, cats and flies as carriers of *Salmonella typhimurium*. *Poultry Sci.* **23**, 456.

MCNEIL, E. & HINSHAW, W. R. (1946). Salmonella fiom Galapagos turtles, a Gila monster and an iguana. *Am. J. vet. Res.* **7**, 62.

MACKERRAS, M. J. (1961). The haematozoa of Australian reptiles. *Aust. J. Zool.* **9**, 61.

MACKERRAS, M. J. (1962). Filarial parasites (Nematoda: Filarioda) of Australian animals. *Aust. J. Zool.* **10**, 400.

MACKEY, J. P. (1955). Salmonellosis in Dar es Salaam. *E. Afr. med. J.* **32**, 1.

MURPHY, W. J. & MORRIS, J. F. (1950). Two outbreaks of gastro-enteritis apparently caused by a paracolon of the Arizona group. *J. infect. Dis.* **86**, 255.

PARKER, R. R. & STEINHAUS, E. A. (1943). *Salmonella enteritidis*: experimental transmission by the Rocky Mountain wood tick *Dermacentor andersoni* Stiles. *Publ. Hlth Rep., Wash.* **58**, 1010.

RAPPAPORT, F., KONFORTI, N. & NAVON, B. (1956). A new enrichment medium for certain salmonellae. *J. clin. Path.* **9**, 261.

REWELL, R. E., TAYLOR, J. & DOUGLAS, S. H. (1948). A new salmonella type (*S. Takoradi*) isolated from a python. *Mon. Bull. Minist. Hlth* **7**, 266.

RUDAT, K.-D., BECK, G., FRANK, W. & RUGOWSKY, G. M. (1966). Über das Vorkommen von Salmonellen bei Reptilien in Zoologischen Gärten. *Pathologia Microbiol.* **29**, 623.

SACHS, A. (1939). Difficulties associated with the bacteriological diagnosis of bacillary dysentery. *Jl R. Army med. Cps* **73**, 235.

STENZEL, W. (1960). Die Sorbosespaltung als Kriterium in der Diagnostik pathogener Enterobacteriaceen. *Zentbl. Bakt. ParasitKde* (Abt. I). Orig. **180**, 19.

J. Hyg., Camb. (1969), **67**, 147
Printed in Great Britain

An outbreak of 'winter vomiting disease' in a university hall of residence

BY JOHN D. CUMMING
University Health Centre, Reading

AND COLIN P. MCEVEDY
Department of Psychological Medicine, The Middlesex Hospital, London, W. 1

(*Received* 1 *August* 1968)

Where feeding is communal, epidemics of vomiting suggest food poisoning. It is usually possible to discover the food and the organism responsible, but in some cases it is not and then the episode tends to get the restrospective label of 'Winter Vomiting Disease'. This disease is thought by many to be due to an airborne virus which acts via the central nervous system; on clinical grounds it has been related to 'Epidemic Vertigo' and 'Epidemic Collapse', for which viral agents have also been postulated (Editorial, 1965). As at least some instances of 'Epidemic Collapse' are really hysterical, the possibility that an hysterical mechanism is involved in 'Winter Vomiting Disease' is worth investigation. An outbreak at Reading provided an opportunity to test this hypothesis.

The epidemic occurred at a Reading University Hall of Residence for Women Students. At the time there were 165 students present. The first two cases vomited at 4.15 p.m. and 5.30 p.m. on 31 January 1967 (day 1). By 8 p.m. 11 girls had been sick and the number rose steadily as the night progressed. At midday on day 2 the figure was 74 of whom two had required admission to the University Health Centre. During the early afternoon of day 2 there were no new cases but that evening and on the night of day 2/day 3 there were a further 12 cases. Five more occurred before the end of the epidemic on day 5.

The late afternoon start of the epidemic suggested that if food poisoning was the cause, lunch was the suspect meal. However, it was soon discovered that quite a number of the affected girls had not eaten lunch in Hall on day 1. A rather smaller number had not eaten dinner and three girls had not eaten either meal.

The outbreak was investigated by the Public Health and Welfare Department of Reading County Borough at the request of one of us (J. D. C.). No pathogens were isolated from specimens of food obtained from the Hall kitchen. No pathogens were seen on direct microscopy of stool samples taken from six of the students most severely affected and none were obtained by either aerobic or anaerobic culture.

From an operational point of view the negative results of the pathological investigations and the failure to obtain a correlation between Hall meals and vomiting put the epidemic in the category of 'Winter Vomiting Disease'. The hypothesis of an hysterical factor in the spread of the vomiting seemed particularly

worthy of investigation in this instance because the two girls admitted to the Health Centre had been in a state of tetany due to overbreathing. Also, there was a strong impression that the cases were geographically clustered within the Hall. Early reports indicated that along some corridors nearly all the girls had been affected whereas in other parts of the Hall and in some of the annexes there had been no cases at all. Such a response by social cells would, if substantiated, fit well with mass hysteria.

It was decided to test the hypothesis of epidemic hysteria by establishing the answers to the following questions:

(1) Did the affected girls have a higher score in neuroticism (N) on the Eysenck Personality Inventory than the unaffected?

(2) Did the affected have a higher frequency of attendance at the University Health Centre prior to the epidemic?

(3) Was there, in fact, a clear geographical clustering of cases?

METHOD

Within 2 weeks of the epidemic questionnaires were given to all the girls, both affected and unaffected, to establish if, when and to what extent they were affected and which Hall meals they had eaten on day 1. At the same time the girls were asked to complete an Eysenck Personality Inventory.

The records of attendance at the University Health Centre and a college room list were used to obtain the answers to questions 2 and 3.

All 165 residents who were given questionnaires completed them.

Table 1. *The distribution of cases by social cells*

Cell	Vomited	Nauseated	Unaffected	% vomited
1. Garden Annexe	21	8	5	60
2. 'Twelve'	5	3	3	45
3. South annexe	6	1	6	50
4. Redlands	7	2	4	55
5. Lydford	7	2	2	65
6. Woodville	9	2	6	55
7. Brierly	8	1	2	70
8. House	4	4	2	40
9*a*. Top corridor	11	0	5	70
9*b*. Middle corridor	8	4	6	45
9*c*. Bottom corridor	5	1	5	45
Total	91	28	46	Av. 55 %

RESULTS

The N scores of the vomiters and non-vomiters are not significantly different. Ninety-one vomiters gave a mean N score of 12·2, and 74 non-vomiters a mean N score of 12·4.

The number of visits to the Health Centre was roughly the same for the two groups: 91 vomiters made 132 visits, or 1·45 visits per head, and 74 non-vomiters made 120 visits, or 1·67 visits per head. The records of 600 women randomly

selected from the whole female undergraduate population yielded a similar figure, the number of visits being 913, or 1·52 per head.

The geographical analysis (Table 1) also gave negative results.

These results effectively dispose of the idea that hysteria played a significant part in the spread of this epidemic of vomiting. Any over-emotional behaviour was a consequence of the epidemic and not a contributory cause.

There is some evidence that the small group of those who felt sick but did not vomit (and who are classed as non-vomiters in the analysis above) did satisfy the first two of the three criteria suggested for an hysterical reaction. Splitting the non-vomiting population into nauseated and unaffected groups we get:

(1)	Nauseated (28)	Mean N score 12·9
	Unaffected (46)	Mean N score 12·0
(2)	Nauseated (28)	66 visits to the Health Centre, i.e. 2·36 a head
	Unaffected (46)	54 visits to the Health Centre, i.e. 1·17 a head

This small element may therefore have contained a proportion of girls whose reaction was a neurotic one suggested by the illness around them. As this would merely be a fringe effect one would not expect it to meet the criterion of geographical concentration.

For the vomiters, who constituted the epidemic proper, we are forced back to an organic explanation and in particular to food poisoning. For this there is positive evidence because the analysis of the questionnaires established a definite relationship between vomiting and eating meals on day 1. Taking the actual times of vomiting given by the group of 71 who ate lunch and/or dinner on day 1 and vomited before midday on day 2, 8 women gave 8 p.m. on day 1 as the time they vomited, 7 women gave 2 a.m. on day 2, while the three next most frequently nominated times had only 5, 4 and 3 nominations. Lunch was served at 1 p.m., dinner at 7 p.m.; the intervals between the first meal and the first peak and between the second meal and the second peak are the same (7 hr.). Plotting out the vomiting times for the 71 cases shows that the distribution is readily analysable as two overlapping responses, the information as to meals eaten being compatible with the time of vomiting in the individual case (Fig. 1). The analysis into two curves is based on the following:

(1) That as no case occurred within 2 hr. of lunch, so no case due to dinner-time poisoning would manifest before 9 p.m. Therefore, all cases prior to 9 p.m. were due to poisoning at lunch.

(2) That the causative agent was the same on both occasions and, as the peaks of the two responses were of the same height, the two response curves must have been identical.

(3) That decay from the peak incidence was exponential, the number of new cases halving in every 2 hr. period.

An additional point in favour of a connexion between eating in Hall and vomiting is the higher proportion of girls missing lunch on day 1, among those who

did not vomit (25 out of 91 = 28 %) as compared to those who did (9 out of 74 = 12 %).

There remain the three cases who did not eat either lunch or dinner on day 1, yet vomited before midday on day 2, and the 17 cases who vomited between late afternoon on day 2 and the evening of day 5. One explanation of these and of the epidemic as a whole would be that there was contamination of the food: (*a*) to a very mild degree at breakfast on day 1, causing 3 cases; (*b*) to a moderate degree at lunch on day 1, causing 35/36 cases; (*c*) to a moderate degree at dinner on day 1, causing 35/36 cases; (*d*) to a moderate degree at lunch on day 2, causing 12 cases; (*e*) to a mild degree on day 3, causing 3 cases; (*f*) to a very mild degree on day 4 and 5, causing 2 cases.

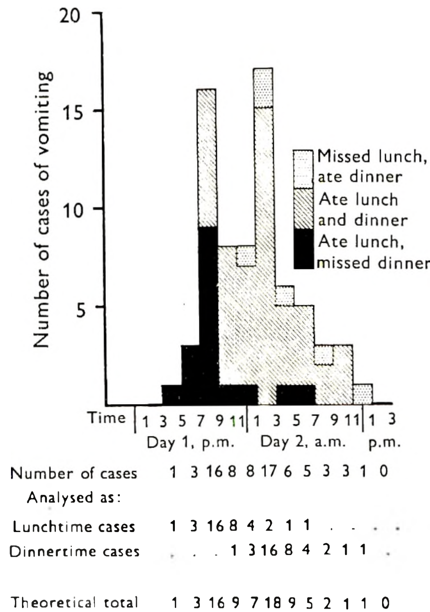


Fig. 1. Time course of the epidemic. Number of women vomiting and meals eaten.

An alternative view would be to regard the mode of spread as 80 % via food but 20 % by contact. The later cases could then be put down to this second form of transmission which would presumably involve a smaller innoculum and a longer incubation period.

DISCUSSION

As all three of the predictions made from the hypothesis of hysterical spread turned out to be incorrect, this hypothesis can be regarded as disproved. Neurotic mechanisms may have played a part (together with subemetic poisoning) among the small group of those who felt sick but did not vomit, but 'Winter Vomiting Disease' is certainly an organic entity.

The hypothesis generally favoured is indeed an organic one—an airborne virus producing its effects via the central nervous system (Miller & Raven, 1936; Gray,

1939; Bradley, 1943; Haworth, Tyrrell & Whitehead, 1956). Food poisoning, it is admitted, cannot be excluded but no one seems to regard it with enthusiasm. The positive results of this study support food poisoning as the mechanism and suggest that it is unrealistic to demand that contamination be limited to a single meal before invoking spread via food. Our postulated agent is, of course, as mysterious as the alternative 'respiratory virus', but we would claim that the portal of entry suggested is inherently more likely in view of the rapid response of the majority of cases and the symptoms of vomiting and, to a less prominent extent, diarrhoea.

The above is written on the somewhat shaky presumption that 'Winter Vomiting' is a true disease entity. As the diagnosis is made on negative evidence it is more than possible that different agents are at work in different outbreaks. The episode described by Haworth *et al.* (1956), with a low attack rate and slow time course (18 cases in a month), certainly fits more readily with a viral theory than the 'explosive' type of epidemic described here. However, as the explosive category appears to include the majority of outbreaks reported in this country the observations in this paper are probably applicable to the syndrome of 'Winter Vomiting Disease' as currently recognized.

SUMMARY

1. An outbreak of 'Winter Vomiting Disease' is described involving women at a University Hall of Residence. Of the 165 women exposed, 74 vomited in the first 24 hr., and 17 more over the next 4 days.

2. Public health investigations were negative.

3. On the hypothesis that the vomiting was at least partly hysterical it was predicted that the affected would have higher N scores than the unaffected on the Eysenck Personality Inventory; that they would have a higher frequency of past attendance at the University Health Centre; and that they would be non-randomly distributed through the Hall and its annexes. All these predictions proved to be incorrect.

4. There is some slight but consistent evidence to suggest that there is a neurotic component in the small group of 28 who felt nauseated but did not vomit.

5. The histogram of time of vomiting has two peaks. It is shown that 71 out of the first 74 cases can be accounted for on the hypothesis of contamination of the food at both lunch and dinner on the first day of the outbreak, with the mean response coming 7 hr. after eating.

6. It is suggested that these results make a food-borne agent a more likely explanation of 'Winter Vomiting Disease' than the currently favoured airborne virus acting via the central nervous system.

The costs involved in the psychiatric side of this investigation were defrayed by a grant from the Clinical Research Committee of the Middlesex Hospital. We would like to thank the Chairman and members of the Committee for making these funds available and for their interest and confidence. We would also like to thank Dr A. W. Beard, Consultant Physician at the Middlesex Hospital for his advice and support.

REFERENCES

BRADLEY, W. H. (1943). Epidemic nausea and vomiting. *Br. med. J.* i, 309.

EDITORIAL. (1965). Winter Vomiting Disease. *Br. med. J.* ii, 953.

GRAY, J. D. (1939). Epidemic nausea and vomiting. *Br. med. J.* i, 209.

HAWORTH, J. C., TYRRELL, D. A. J. & WHITEHEAD, J. E. M. (1956). 'Winter Vomiting Disease' with meningeal involvement. *Lancet* ii, 1152.

MILLER, R. & RAVEN, M. (1936). Epidemic nausea and vomiting. *Br. med. J.* i, 1242.

J. Hyg., Camb. (1969), **67**, 153
Printed in Great Britain

Factors affecting lecithinase activity and production in *Clostridium welchii**

BY M. NAKAMURA, JUDITH A. SCHULZE AND W. R. CROSS†
Department of Microbiology, University of Montana, Missoula, Montana 59801

(*Received* 6 *August* 1968)

INTRODUCTION

Interest in *Clostridium welchii* food poisoning was generated by the work of Hobbs *et al.* (1953). In order to explain the pathogenicity of *Cl. welchii* in food poisoning, Nygren (1962) proposed that phosphorylcholine, the end product of the action of lecithinase upon lecithin, was responsible for the symptoms of food poisoning. On the other hand, Dische & Elek (1957) and Dack (1947) have suggested that large numbers of living organisms were responsible for clinical disease.

Although the role of lecithinase produced by *Cl. welchii* in food poisoning is unclear, Weiss & Strong (1967) and Nakamura & Cross (1968) studied the lecithinase production by strains of *Cl. welchii* isolated from food-borne outbreaks.

Collee, Knowlden & Hobbs (1961) reported that optimum growth of *Cl. welchii* occurred at temperatures between 43° and 47° C. Boyd, Logan & Tytell (1948) obtained maximum growth of this organism after 16–20 hr. of incubation. The optimum temperature for growth of *Cl. perfringens* may not be identical with the optimum temperature for lecithinase production.

The lecithinase of *Cl. welchii* is resistant to inactivation by heat. Macfarlane & Knight (1941) reported that lecithinase preparations retained 45 % of their activity when exposed to 100° C. Smith & Gardner (1949) found that lecithinase was greatly inactivated at 65° C. but could be partially reactivated by further heating to 100° C. They suggested that enzymically inactive complexes linked by calcium ions were formed at 65° C. However, at 100° C. these complexes dissociated with the liberation of active lecithinase. Purified lecithinase, but not culture filtrates, were heat-sensitive (Kushner, 1957). Weiss & Strong (1967) reported that lecithinase retained more activity after heating at 100° C. than at 75° C. They noted that the degree of heat resistance of lecithinase was somewhat dependent upon the strain of *Cl. welchii* studied.

Optimum lecithinase activity was observed at pH 7·0–7·6 (Macfarlane & Knight, 1941). However, Smith & Gardner (1949) demonstrated that lecithinase activity occurred at a pH range of 5·0–9·0.

Very little is known about the nutritional factors that stimulate lecithinase production by *Cl. welchii.*

* This study was supported by United States Public Health Service grant UI-00291-03 from the National Center for Urban and Industrial Health.

† U.S. Public Health Service predoctoral research fellow (GM-39,036-01) supported by the National Institute of General Medical Sciences, Public Health Service.

In this paper we describe studies on the effect of time and temperature of incubation on lecithinase production by *Cl. welchii*. We also describe experiments on the effects of heat and pH on lecithinase activity. Studies on the stimulation of lecithinase production by lecithin, in a chemically defined medium, are also described.

GENERAL METHODS

Assay of lecithinase activity

The lecithinase activity was assayed using the lecithovitellin reaction method of van Heyningen (1941) as modified by Nakamura & Cross (1968). In the modified method the lecithinase activity is reported as μg./ml. of lecithinase in culture filtrates as determined from a standard assay curve using commercial purified lecithinase (Nutritional Biochemicals Corp., Cleveland, Ohio).

Some of the data on the lecithinase activity are recorded in terms of changes in optical density produced as a result of the effect of lecithinase upon the egg-yolk-saline substrate. The readings were made with a Coleman Junior, Model 6C, spectrophotometer with the wavelength set at 650 mμ.

Culture media

Complex medium for lecithinase production by *Cl. welchii* consisted of trypticase, 20 g.; yeast extract, 5 g.; soluble starch, 2·5 g.; sucrose, 1 g.; $K_2HPO_4.3H_2O$, 1 g.; $MgSO_4.7H_2O$, 0·1 g.; $Fe_2(SO_4)_3.nH_2O$, 0·1 g.; distilled water, 1000 ml. The pH was adjusted to 6·8.

Lecithinase production was studied in a chemically defined medium. The medium was commercially available tissue-culture medium NCTC 109 (Grand Island Biological Company, Grand Island, N.Y.) to which synthetic glycylglycine (Nutritional Biochemicals Corp., Cleveland, Ohio) was added in varying concentrations. The pH of the medium after pressure filtration was 7·2.

EXPERIMENTAL STUDIES

The effect of temperature and length of incubation on lecithinase activity of *Clostridium welchii*

Weiss & Strong (1967) incubated cultures of *Cl. welchii* at 37° C. and found that lecithinase activity reached a peak between 2–4 hr. and then decreased rapidly to a minimum in 6–10 hr.

In this study we have extended the time of incubation and varied the temperature of incubation in order to determine the lecithinase activity under various conditions.

Methods

Three strains of type A *Cl. welchii* were used in these studies. Strain UMJS-12 was isolated from human faeces. Strain UMJS-39 was isolated from soil. Strain UMKK-29 was isolated from cold cut meat. The strains were identified on the basis of morphological and biochemical characteristics.

Screw-capped test-tubes containing 10 ml. of complex medium for lecithinase production were inoculated with 1 ml. of an 18–24 hr. thioglycollate broth culture of each strain. The tubes were incubated at 10°, 30°, 46°, and 52° C. The culture filtrates were assayed for lecithinase activity after 2, 5, 10, 30 and 60 hr. of incubation.

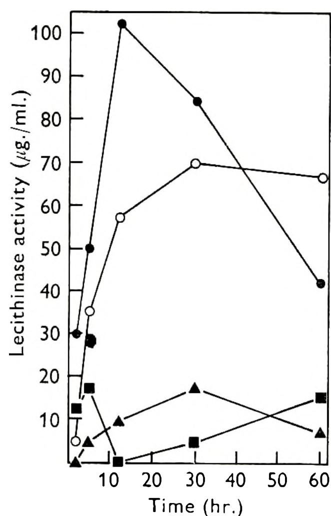


Fig. 1

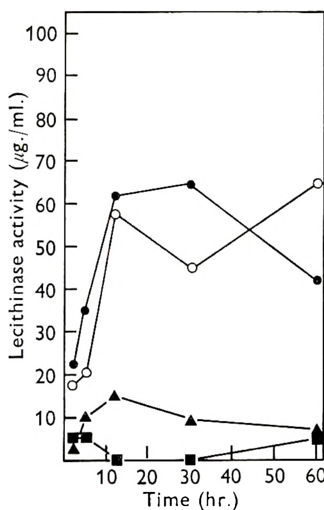


Fig. 2

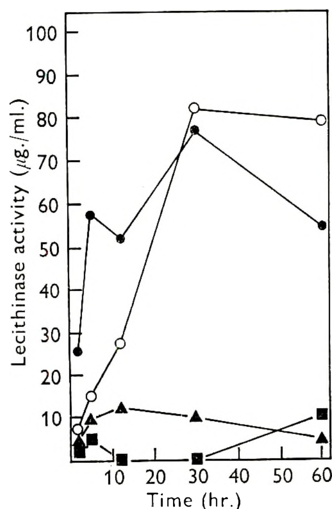


Fig. 3

Fig. 1. The effect of time and temperature of incubation on the lecithinase activity of *Cl. welchii* strain UMJS-12. ●—●, 46° C.; ○—○, 30° C.; ■—■, 52° C.; ▲—▲10° C.

Fig. 2. The effect of time and temperature of incubation on the lecithinase activity of *Cl. welchii* strain UMKK-29. ●—●, 46° C.; ○—○, 30° C.; ▲—▲, 10° C.; ■—■, 52, C.

Fig. 3. The effect of time and temperature of incubation on the lecithinase activity of *Cl. welchii* strain UMJS-39. ●—●, 46° C.; ○—○, 30° C.; ▲—▲, 10° C.; ■—■, 52° C.

Results

The lecithinase activity of *Cl. welchii* was time- and temperature-dependent. The results from the three strains studied are presented in Figs. 1–3. The optimum time and temperature of incubation for lecithinase production varied slightly with each strain. Strain UMJS-12 produced maximum lecithinase activity after 12 hr. of incubation at 46° C., whereas strain UMKK-29 produced maximum lecithinase activity after 12–30 hr. of incubation at this temperature. Strain UMJS-39 produced maximum lecithinase activity after 30–60 hr. of incubation at a temperature of 30° C. Lecithinase activity was negligible at 10° and at 52° C., the lower and upper limits for growth of *Cl. welchii*. There were no viable cells present in cultures incubated at 52° C. However, small amounts of lecithinase were detected. It is possible that the cells used in the inoculum possessed lecithinase which was released into the medium after cell lysis.

The lecithinase activity in cultures incubated at 30° C. appeared to be relatively

stable. On the other hand, the lecithinase activity in cultures incubated at 46° C. reached a maximum and then declined upon continued incubation.

EFFECT OF HEAT ON LECITHINASE ACTIVITY

In this study we have examined the susceptibility of commercial lecithinase and lecithinase in culture filtrates to heat.

Methods

Strain PB 6 H of *Cl. welchii* was grown for 5 hr. at 46° C. in the complex medium. Cell-free culture filtrates were obtained by centrifuging each culture for 30 min. at 4000 rev./min. The supernatant fluid was passed through a sterilizing filter pad. Each filtrate preparation was adjusted to pH 7·2 by the addition of 1 N-NaOH. These filtrate preparations served as the source of crude lecithinase. Two stock solutions of the commercial lecithinase were prepared. One stock solution was prepared by dissolving 100 μg./ml. of enzyme in 0·05 M Tris (Tris(hydroxymethyl)-aminomethane) buffer, pH 7·2. The second solution was prepared by dissolving 100 μg./ml. of the enzyme in the complex medium, pH 7·2.

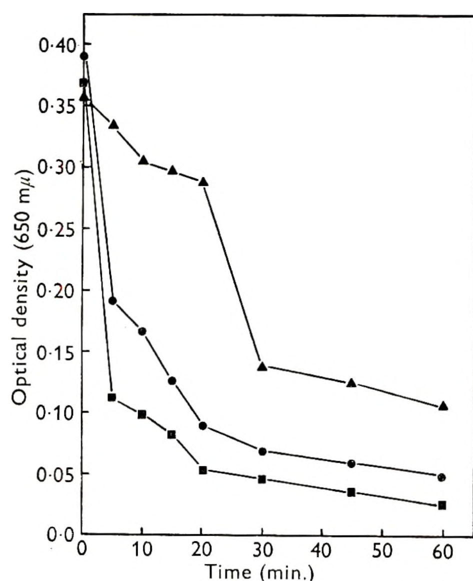


Fig. 4

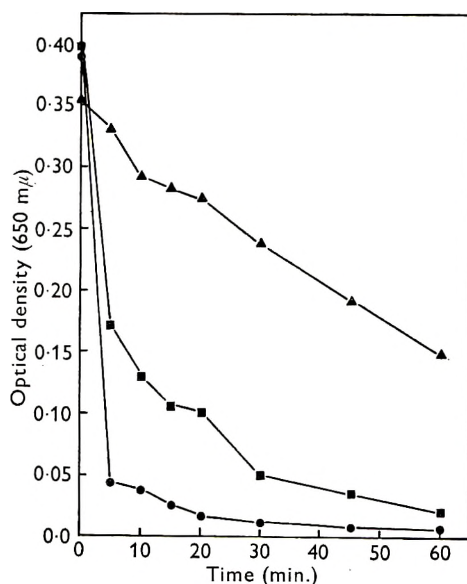


Fig. 5

Fig. 4. The inactivation of lecithinase by heat (60° C.). ●—●, Lecithinase dissolved in 0·05 M tris buffer. ▲—▲, Culture filtrate of strain PB 6 H. ■—■, Lecithinase dissolved in medium for α-toxin production.

Fig. 5. The inactivation of lecithinase by heat (90° C.). ●—●, Lecithinase dissolved in 0·05 M tris buffer, ▲—▲, Culture filtrate of strain PB 6 H. ■—■, Lecithinase dissolved in medium for α-toxin production.

Five ml. of each lecithinase preparation were pipetted into 16 × 125 mm. screw-capped test-tubes. The tubes were placed in a water bath preheated to the desired temperature. After heating for the desired length of time, the tubes were cooled in an ice-bath. One ml. of each preparation was assayed for lecithinase activity.

Results

The lecithinase present in culture filtrates of *Cl. welchii* was relatively heat-resistant. The preparation lost only 20 % of its original activity when heated for 20 min. at 60° C. Figure 4 shows the decline in enzymic activity as a function of heating time. A marked decrease in activity resulted upon heating beyond 20 min. Commercial lecithinase, dissolved in complex medium and buffer, was more rapidly inactivated than the enzyme in the culture filtrate.

Figure 5 shows the effect of a higher temperature, 90° C., upon lecithinase. The enzyme inactivation pattern was similar to that at 60° C. Commercial lecithinase was inactivated much more rapidly than the lecithinase in the culture filtrate.

Effect of pH on lecithinase and its activity

Gale & van Heyningen (1942) reported that in the absence of glucose lecithinase is produced when the pH of the medium lies between 5·5 and 7·0, with a maximum production at pH 6·0. In the presence of glucose the maximum production of lecithinase took place at a pH of 7·0–7·5.

In this study we examined the effects of pH on commercial lecithinase and the effects of pH on the hydrolysis of lecithovitellin by this enzyme. We studied the effects of pH on the growth and lecithinase activity of *Cl. welchii*, strain BP6K, a classical gas-gangrene strain, as well as on strain Hobbs 3, isolated from boiled salt beef (originally National Collection of Type Cultures 8239).

Methods

Commercial lecithinase was dissolved in distilled water to yield a final concentration of 100 μg/ml. The pH of the solvent was adjusted to the desired pH by the addition of 0·1 N-HCl or 0·1 N-NaOH. After exposing the enzyme preparations to the various pH values for 1 hr., the pH of each sample was readjusted to pH 7·0. One ml. of each solution was assayed for lecithinase activity. Lecithinase exposed to pH 7·0 was used as the control (100 % activity).

The effects of pH on the hydrolysis of lecithovitellin (the substrate) by lecithinase were determined by adjusting the pH of the reaction menstruum from pH values 1–9. In place of the Tris buffer, HCl or NaOH were added to adjust the pH. The control consisted of the optical density produced at pH 7·0, which was arbitrarily defined as 100 % activity.

The enzymic activity remaining after exposure to various pH values was calculated as a percentage by dividing the optical density produced in the experimental tubes by the optical density of the control and multiplying these values by 100.

The effect of pH on growth and lecithinase production in the growth medium was studied. Standard plate count methods were used to enumerate the bacterial population.

Results

Table 1 summarizes the effect of pH on commercial lecithinase. Exposure to pH values of 1–3 for 1 hr. completely inactivated the enzyme. At pH 5 half of the activity was lost. Maximum enzyme activity remained after exposure to pH values of 6–9. After exposure to pH 10, 63·2 % of the activity was lost.

Table 1. *Effect of pH on the activity of commercial purified lecithinase*

pH	Optical density (absorbance at 650 mμ)	Activity remaining (%)
1·0	0 000	0
2·0	0·000	0
3·0	0·000	0
4·0	0·010	3·0
5·0	0·170	50·0
6·0	0·320	97·0
7·0*	0·340	100·0
8·0	0·333	98·0
9·0	0·333	98·0
10·0	0·125	36·8

* Defined as 100% activity.

Table 2. *Effect of pH on the hydrolysis of lecithovitellin (egg yolk saline) by lecithinase*

pH	Optical density (absorbance at 650 mμ)	Activity compared to control (%)
1·0	0·000	0
2·0	0·000	0
3·0	0·000	0
4·0	0·000	0
5·0	0·000	0
6·0	0·072	27·6
7·0*	0·260	100·0
8·0	0·210	80·7
9·0	0·030	11·5
10·0	0·008	3·0

* Defined as 100% activity.

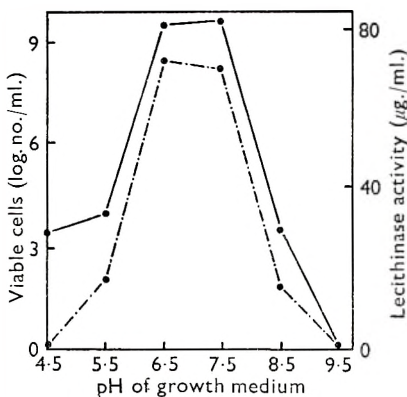


Fig. 6

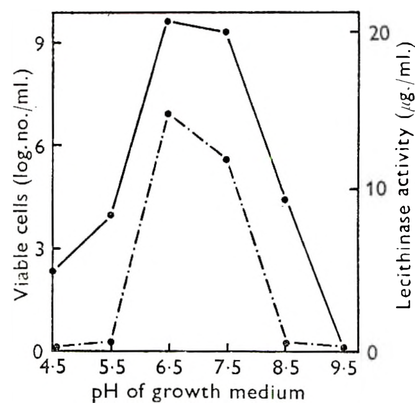


Fig. 7

Fig. 6. The effects of pH on the growth and lecithinase production in *Cl. welchii* strain BP6K. ●—●, Log. no. of cells/ml. ●-·-·●, Lecithinase activity (μg./ml.).

Fig. 7. The effects of pH on the growth and lecithinase production in *Cl. welchii* strain Hobbs 3. ●—●, Log. no. of cells/ml. ●-·-·●, Lecithinase activity (μg./ml.).

The effect of pH on the enzymic reaction is shown in Table 2. The hydrolysis of the lecithovitellin was completely inhibited at pH values of 1–5. Although some hydrolysis occurred at pH 6·0 and 9·0, the optimum pH for the reaction to occur appears to be around pH 7–8.

The effects of pH on growth and lecithinase production during growth are shown in Figs. 6 and 7. Although growth occurred over a wider pH range, lecithinase production was more restricted by pH changes. However, the optimum pH for growth paralleled that for lecithinase production. Strain BP6K produced measurable lecithinase at pH values 5·5–8·5, whereas strain Hobbs 3 produced lecithinase only at pH values 6·5–7·5.

Effect of synthetic lecithin on lecithinase production

The production of large amounts of lecithinase by *Cl. welchii* in a chemically defined medium was recently reported (Nakamura, Cook & Cross 1968). A number of synthetic dipeptides stimulated lecithinase production in the defined medium. A study of the possible precursors of lecithinase synthesis may yield vital information regarding the nutritional conditions necessary for optimum lecithinase production.

Gordon, Turner & Dmochowski (1954) reported that lecithin inhibited the haemolytic activity of lecithinase. Although Adams, Hendee & Pappenheimer (1947) found that lecithin stimulated lecithinase production, their experiments were performed in a complex non-synthetic medium.

In this study we describe the stimulatory activity of lecithin upon lecithinase production by *Cl. welchii* in a chemically defined medium.

Table 3. *The effects of lecithin on lecithinase production by* Clostridium welchii, BP6K, *grown in a chemically defined medium supplemented with glycylglycine*

Concentration of glycylglycine (mg./ml.)	Lecithinase activity (μg./ml.) — Concentration of lecithin 0·0	0·1 mg./ml.
0·0	0·0	0·0
0·01	67·0	90·0
0·05	64·0	82·0
0·10	30·0	60·0
0·20	10·0	20·0

Methods

Synthetic lecithin (L-α-lecithin) obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, was added at a concentration of 0·1 mg./ml. to the chemically defined medium fortified with glycylglycine. Experiments were also performed in media devoid of glycylglycine in order to determine the importance of this dipeptide in lecithinase production.

Strain BP6K of *Cl. welchii* was maintained for over twenty subcultures in the defined medium in order to eliminate the possibilities that carry-over material from the complex medium might stimulate lecithinase production. Twenty-four hr. cultures were assayed for lecithinase activity.

Results

Lecithin stimulated the production of lecithinase. However, the stimulatory activity was dependent upon the concentration of glycylglycine present. The data are presented in Table 3. In the absence of glycylglycine lecithin did not stimulate lecithinase production. There was an optimum concentration for both glycylglycine and lecithin in the production of lecithinase.

DISCUSSION

Quantitatively, lecithinase production by *Cl. welchii* is dependent upon the strain, the medium, the culture conditions, pH, temperature of incubation, and the length of incubation. One of the strains, UMJS-12, in our study produced more lecithinase at 46° than at 30° C. The other two strains produced comparable amounts of lecithinase at both of these temperatures. This reflects the variation in the ability of different strains to produce lecithinase at different temperatures.

Roberts (1957) reported that there was less lecithinase produced when the cultures were incubated at 43° C. compared to 37° C. However, he measured lecithinase activity by haemolysis, whereas in our work we have used the lecithovitellin reaction to measure lecithinase activity. In other words, the activity assayed may indicate different kinds of lecithinase levels. In fact, several investigators have suggested the possibility that the haemolytic activity due to alpha toxin may be due to a component separate from the lecithinase which produces the lecithovitellin reaction (Miles & Miles, 1950; Matsumoto, 1961).

It was reported by Weiss & Strong (1967) that the lecithinase activity of some strains decreased rapidly after reaching a maximum after 4–6 hr. of incubation. In our work the lecithinase activity remained relatively stable or decreased only slightly after maximum activity was observed. Our data indicated that the lecithinase activity in older cultures (30–60 hr.) was greater than the lecithinase activity in young actively growing cultures (5 hr.). Gale & van Heyningen (1942) also reported that the production of lecithinase varied with the age of the culture. The amount of lecithinase produced was small in young cultures and increased to a maximum at the time when active cell division ceased. It is possible that the composition of the medium used for the growth and production of lecithinase plays an important role in the stability of the lecithinase after it is produced. In culture media deficient in nutrients, it is possible that the lecithinase, which is a protein, is broken down and metabolized.

A portion of the lecithinase activity in older cultures may have been due to the lysis of the cells which may release the lecithinase into the culture medium. This is a possibility because it was reported (M. Nakamura & W. R. Cross, unpublished data) that some strains of *Cl. welchii* retain over 50 % of the lecithinase within the cell.

Lecithinase in culture filtrates was more resistant to inactivation by heat than purified commercial lecithinase. A possible explanation is that the crude lecithinase in culture filtrates may differ from that of the purified preparation. Purified lecithinase heated in the sterile complex medium retained its heat sensitivity, suggesting that the complex medium itself did not protect the enzyme against

heat. Therefore, another possibility is that metabolites produced by the growing *Cl. welchii* may have protected the enzyme against heat. Conflicting data were presented by Kushner (1957) who reported that the lecithinase activity, using purified material, was almost completely destroyed after 5 min. at 100° C. On the other hand, he found that lecithinase in the culture filtrate was resistant to decomposition under similar conditions.

Although our data indicate that less inactivation of the enzyme occurred at 90° C. compared to 60° C. the differences were not particularly significant. Smith & Gardner (1949) suggested that the lower temperatures increased inactivation possibly because of the formation of enzymically inactive complexes. They suggested that calcium ions added during the heating procedure mediated the formation of inactive complexes. In our study only trace amounts of calcium ions were present. This might account for the similarity in the heat inactivation patterns we observed at the two temperatures.

It was reported that lecithinase was more resistant to heat at alkaline pH values than at acid pH values (Macfarlane & Knight, 1941; Smith & Gardner, 1949). The decrease in heat-resistance in an acid environment may have been due to partial inactivation of the enzyme by the acid.

Lecithinase was readily inactivated by acid pH, but was more resistant to alkaline pH values. Others reported that the lecithinase was most stable at pH 6, losing activity rapidly at pH 7, and very rapidly above pH 8 (Adams & Hendee, 1945). The pH for optimal hydrolytic reaction of the lecithovitellin was between pH values of 7 and 8. The effect of pH may be upon the substrate as well as on the enzyme system. This possibility is supported by the observation that lecithinase retained 50 % of its activity when exposed to pH 5·0 for 1 hr., but no detectable hydrolysis occurred when the enzyme reaction was measured at this pH.

Our data do not permit us to speculate much regarding the reasons why lecithin stimulated lecithinase production in the presence of glycylglycine. However, one possibility is that additional substrate, when present in the culture medium, stimulated enzyme formation because lecithinase is a partially inducible enzyme. The addition of lecithin increased lecithinase production by 50–100 %. However, this was possible only if the concentration of glycylglycine was optimal.

The data presented in this study suggest that conflicts in the published literature and variations in the results reported may be due to differences in methodology and strain of *Cl. welchii* used by various investigators.

SUMMARY

A variety of factors that affect lecithinase activity and lecithinase production by *Cl. welchii* were studied. The lecithinase activity was time and temperature dependent. The optimum temperature varied from 30° to 46° C. according to the strain of *Cl. welchii* employed. Maximum lecithinase activity was produced after 12–60 hr. There was considerable strain variation. This could easily account for the differences in the published data.

Commercial purified lecithinase was more readily destroyed by heat than the lecithinase produced by *Cl. welchii* in culture media. The enzyme inactivation

pattern at 60° C. was similar to that at 90° C. This is not in agreement with the reports of others who found that less enzyme was inactivated at the higher temperatures.

Acid pH values completely inactivated the enzyme. However, alkaline pH values did not significantly destroy the enzyme. The lecithovitellin reaction was completely inhibited at pH values of 1–5. The optimum pH for the reaction was around pH 7–8.

The production of lecithinase was dependent upon the pH of the culture medium. One strain produced measurable lecithinase at pH values of 5·5–8·5, whereas another strain produced lecithinase only at pH values 6·5–7·5.

Lecithin stimulated the production of lecithinase in a chemically defined medium.

REFERENCES

ADAMS, M. H. & HENDEE, E. D. (1945). Methods for the production of the alpha and theta toxins of *Clostridium welchii*. *J. Immun.* **51**, 249.

ADAMS, M. H., HENDEE, E. D. & PAPPENHEIMER, A. M. (1947). Factors involved in production of *Clostridium welchii* alpha toxin. *J. exp. Med.* **85**, 701.

BOYD, M. J., LOGAN, M. A. & TYTELL, A. A. (1948). The growth requirements of *Clostridium perfringens* (*welchii*) BP6K. *J. biol. Chem.* **174**, 1013.

COLLEE, J. G., KNOWLDEN, J. A. & HOBBS, B. C. (1961). Studies on the growth, sporulation and carriage of *Clostridium welchii* with special reference to food poisoning strains. *J. appl. Bact.* **24**, 326.

DACK, G. M. (1947). Problems and errors in assigning causes of bacterial food poisoning. *Am. J. publ. Hlth* **37**, 360.

DISCHE, F. E. & ELEK, S. D. (1957). Experimental food-poisoning by *Clostridium welchii*. *Lancet* ii, 71.

GALE, E. F. & VAN HEYNINGEN, W. E. (1942). The effect of the pH and the presence of glucose during growth on the production of α and θ toxins and hyaluronidase by *Clostridium welchii*. *Biochem. J.* **36**, 624.

GORDON, J., TURNER, G. C. & DMOCHOWSKI, L. (1954). The inhibition of the alpha and theta toxins of *Clostridium welchii* by lecithin. *J. Path. Bact.* **67**, 605.

HOBBS, B. C., SMITH, M. E., OAKLEY, C. L., WARRACK, G. H. & CRUICKSHANK, J. C. (1953). *Clostridium welchii* food poisoning. *J. Hyg., Camb.* **51**, 75.

KUSHNER, D. J. (1957). An evaluation of the egg-yolk reaction as a test for lecithinase activity. *J. Bact.* **73**, 297.

MACFARLANE, M. G. & KNIGHT, B. C. J. G. (1941). The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. *Biochem. J.* **35**, 884.

MATSUMOTO, M. (1961). Studies on phospholipids. II. Phospholipase activity of *Clostridium perfringens* toxin. *J. Biochem.* **49**, 23.

MILES, E. M. & MILES, A. A. (1950). The relation of toxicity and enzyme activity in the lecithinases of *Clostridium bifermentans* and *Clostridium welchii*. *J. gen. Microbiol.* **4**, 22.

NAKAMURA, M., COOK, J. A. & CROSS, W. R. (1968). Lecithinase production by *Clostridium perfringens* in chemically defined media. *Appl. Microbiol.* **16**, 1420.

NAKAMURA, M. & CROSS, W. R. (1968). The lecithinase (α toxin) activity of strains of *Clostridium perfringens*. *Proc. Soc. exp. Biol. Med.* **127**, 719.

NYGREN, N. (1962). Phospholipase C-producing bacteria and food poisoning. An experimental study on *Clostridium perfringens* and *Bacillus cereus*. *Acta path. microbiol. scand.*, suppl. no. 160.

ROBERTS, J. E. (1957). Toxin production by *Clostridium perfringens*. I. Synthesis of α- and θ-hemolysins, lecithinase and proteinase in a synthetic medium. *J. Bact.* **74**, 439.

SMITH, L. DES. & GARDNER, M. V. (1949). The anomalous heat inactivation of *Clostridium perfringens* lecithinase. *Archs Biochem.* **25**, 54.

VAN HEYNINGEN, W. E. (1941). The biochemistry of the gas gangrene toxins. I. Estimation of the α toxin of *Cl. welchii*, type A. *Biochem. J.* **35**, 1246.

WEISS, K. F. & STRONG, D. H. (1967). Some properties of heat-resistant and heat-sensitive strains of *Clostridium perfringens*. I. Heat resistance and toxigenicity. *J. Bact.* **93**, 21.

J. Hyg., Camb. (1969), **67**, 163
Printed in Great Britain

Haemolytic activity of the alpha and theta toxins of *Clostridium welchii**

BY JUDITH A. SCHULZE† AND M. NAKAMURA
Department of Microbiology, University of Montana, Missoula, Montana 59801

(*Received* 20 *August* 1968)

INTRODUCTION

Clostridium welchii type A produces several toxic substances which are lethal, necrotizing, and haemolytic. The lethal and necrotizing properties of this organism have been studied extensively. However, little is known regarding the haemolytic activity of *Cl. welchii*. It is difficult to speculate on the mechanism of haemolysis caused by *Cl. welchii* until the biochemistry and mechanism of the general phenomenon of haemolysis is elucidated.

Of the twelve recognized toxins produced by *Cl. welchii*, only three, alpha, delta and theta toxins, are haemolytic. Since type A strains do not produce the delta toxin, the haemolytic action of type A strains is limited to the activity of alpha and theta toxins. The haemolytic activities of these two toxins are easily recognized; the alpha toxin produces partial haemolysis, whereas the theta toxin produces a complete haemolysis. Non-haemolytic strains of *Cl. welchii* were brought to attention when Hobbs *et al.* (1953) showed that the English 'food poisoning' strains were typically non-haemolytic.

Alpha toxin has been shown by Macfarlane & Knight (1941) to be closely associated, and probably identical, with the enzyme lecithinase found in toxic filtrates of *Cl. welchii*. They suggested that the lecithinase was responsible for the lysis of red cells. The haemolytic activity of the toxin is due to its ability to hydrolyze phospholipid-protein complexes on the surface of susceptible erythrocytes (Macfarlane & Knight, 1941).

Neill (1926) described an oxygen-labile haemolysin present in culture filtrates of *Cl. welchii*. The haemolytic activity of this substance was inactivated by oxidation, and could be restored by the addition of a reducing agent. This oxygen-labile haemolysin was later shown to be identical with theta toxin (Todd, 1941). Although the studies of Todd (1941) and Roth & Pillemer (1955) strongly suggest that this toxin exhibits enzymic properties, no specific substrate has been identified.

Strains of *Cl. welchii* type A have been subdivided into two groups—classical and food-poisoning strains (Hobbs, 1965; Brooks, Sterne & Warrack, 1957). Haemolytic activity is one of the criteria which Hobbs has used to distinguish these two groups. The classical gas-gangrene strains possess heat-sensitive spores, are

* This study was supported by United States Public Health Service grant UI-00291-03 from the National Center for Urban and Industrial Health.

† Present address: Kenosha Memorial Hospital, Kenosha, Wisconsin 53140.

II-2

β-haemolytic on sheep blood agar, and produce relatively large amounts of alpha toxin. The food-poisoning strains produce small amounts of alpha toxin but no theta toxin, are heat-resistant and are non- or α-haemolytic on horse-blood agar (Hobbs, 1965). Other workers (Dische & Elek, 1957; Hauschild & Thatcher, 1967) have suggested that food-poisoning strains may be heat-sensitive as well as heat-resistant, and may vary considerably in their haemolytic activity and formation of alpha toxin. Recently Sutton & Hobbs (1968) reported on five outbreaks of food poisoning caused by heat-sensitive strains of *Cl. welchii*. They suggested that perhaps heat-sensitive strains may be implicated in food poisoning outbreaks with unknown aetiology.

Although haemolysis is used as a criterion for the identification of type A strains of *Cl. welchii* the species of red blood cells and the kind of haemolysis observed is often not indicated. The conditions for determining the haemolytic patterns exhibited by *Cl. welchii* have not been standardized. Haemolysis observed on blood agar plates may be influenced by a number of variables: erythrocytes from different animal species, growth conditions of the organism, temperature, and conditions under which the haemolysis is observed. In order to categorize strains of *Cl. welchii* as to haemolytic pattern, it is important to be aware of the range of variations that may be observed. We studied the haemolytic activity of different strains of *Cl. welchii* using erythrocytes from four different animal species in order to determine the relative value of each blood in the detection of haemolysis, as well as to investigate the comparative haemolytic activities of each strain. In addition, the haemolytic activity was compared with the lecithinase activity of *Cl. welchii*.

MATERIALS AND METHODS

Strains of Clostridium welchii

Thirty strains of *Cl. welchii* were employed in this study. All strains were isolated at the University of Montana, Missoula, Montana, from human faeces, soil, and foods. The strains were identified on the basis of morphological and biochemical characteristics.

Lecithinase activity

The lecithinase activity of *Cl. welchii* was assayed by the lecithovitellin method of van Heyningen (1941) as modified by Nakamura & Cross (1968).

Haemolytic activity of Cl. welchii

The haemolytic activity of each strain was assayed by observing haemolysis on 5·0 % blood agar plates. Defibrinated horse, sheep, and rabbit bloods were obtained from Colorado Serum Company, Denver, Colorado; the human blood was drawn in the laboratory and added directly to the medium without the use of an anticoagulant. Actively growing thioglycollate broth cultures (5 hr. at 46° C.) of each strain were centrifuged and the cells washed three times in saline. The cells were suspended in saline and diluted with sterile 0·1 % peptone water in order to obtain approximately 10–50 colonies per plate. After anaerobic incubation at 37° C. for 18 hr., the plates were placed at 5° C. under aerobic conditions for 4–6 hr. to allow

full development of the haemolytic activity of the alpha toxin, a hot–cold lysin. The diameters of ten colonies of each strain on each type of blood were measured using a vernier caliper with an accuracy of 0·1 mm. (General Hardware Mfg., Inc., New York, New York). Haemolytic zones which circumscribed the colony were measured.

Theta toxin produced a zone of complete clearing of blood around the colony. Microscopic examination indicated that all red blood cells were lysed in this area. Alpha toxin produced partial haemolysis which resulted in a zone of discoloration surrounding the colony. In this area of haemolysis only partial lysis of the red cells was observed microscopically.

The effect of temperature and length of incubation on haemolysis by *Cl. welchii* was determined. After an 18 hr. initial incubation period at 37° C. the plates were incubated further aerobically at 37°, 46°, and 52° C. The diameters of each colony and zones of haemolysis were measured periodically for 24 hr.

Haemolytic activity of commercial lecithinase

The haemolytic activity of commercial lecithinase (Nutritional Biochemicals Corporation, Cleveland, Ohio) upon horse, sheep, rabbit and human blood was studied. The lecithinase was diluted in 0·05 M Tris buffer, pH 7·2, in order to obtain concentrations of 20, 40, 80, 100 and 200 μg./ml. An Ouchterlony die (Shandon Scientific Co., London) was used to make wells with a diameter of 3·5 mm. in the blood agar plates. A 1 ml. tuberculin syringe with a 25-gauge needle was used to dispense the lecithinase solutions. One drop of each concentration was added to the wells in the blood agar plates. One drop approximated 0·008 ml. The plates were then incubated at 37°, 46° and 52° C. The diameter of each zone of haemolysis was measured periodically for 24 hr.

The effect of heat on the haemolytic activity of commercial lecithinase was determined. Three tubes containing 100 μg./ml. solution of lecithinase in Tris buffer were placed in water baths preheated to 50°, 60° and 70° C., respectively. Samples were removed after 2, 5, 10, 20, 30, 45 and 60 min. of heating. Unheated samples were used as controls. One drop of each sample was dispensed into wells on sheep blood agar plates. The plates were incubated at 37° C. for 24 hr., and then held for several hours at 5° C. The diameters of haemolysis were measured using a vernier calipers.

RESULTS

Lecithinase activity

The lecithinase activity of thirty strains of *Cl. welchii* is enumerated in Table 1. The mean lecithinase activity of the ten strains isolated from food was $43·5 \pm 22·1$ μg./ml. whereas the mean lecithinase values of the soil and faecal strains were $67·0 \pm 28·3$ and $65·0 \pm 31·5$ μg./ml., respectively.

Haemolytic activity of Cl. welchii

The haemolytic activity of twenty-nine strains of *Cl. welchii* on horse, sheep, rabbit, and human blood is shown in Table 2. Most of the strains produced suf-

ficient theta toxin to produce lysis on all four types of blood. Seventy-nine per cent of the strains produced theta toxin haemolysis on horse blood, 85% on sheep blood, 90% on human blood, and 100% on rabbit blood. The areas of haemolysis produced on rabbit blood were larger than those produced on the other bloods. Therefore, rabbit blood may be considered the ideal blood for the detection of the theta toxin of *Cl. welchii*.

Table 1. *Lecithinase activity of* Clostridium welchii *isolated from human faeces, soil, and food*

Human faeces		Soil		Food	
Strain	μg. toxin/ml. culture filtrate*	Strain	μg. toxin/ml. culture filtrate*	Strain	μg. toxin/ml. culture filtrate*
UM 2	105·0	UM 31	80·0	UM 7	47·5
UM 3	57·5	UM 32	67·5	UM 17	82·5
UM 5	90·0	UM 33	20·0	UM 19	50·0
UM 6	80·0	UM 34	102·5	UM 26	30·0
UM 8	47·4	UM 35	67·5	UM 29	15·0
UM 9	57·5	UM 36	22·5	UM 46	77·5
UM 10	45·0	UM 37	127·5	UM 48	40·0
UM 12	45·0	UM 38	55·0	UM 52	42·5
UM 13	117·5	UM 39	62·5	UM 54	40·0
		UM 43	45·0	UM 55	10·0

* Mean of two determinations.

Only 3 out of 29 (9·6%) strains produced sufficient amounts of alpha toxin to lyse horse blood. These three strains were highly toxigenic (over 100 μg. alpha toxin per ml.) confirming the work of Hall, Angelotti, Lewis & Foter (1963), who reported that a higher concentration of alpha toxin was required to lyse horse erythrocytes. Thirty-eight per cent of the strains produced alpha toxin haemolysis on rabbit blood, 76% on human blood, and 93% on sheep blood. Since sheep erythrocytes are the most susceptible to lysis by alpha toxin, sheep blood should be considered to be the blood of choice for the detection of haemolysis due to alpha toxin. Hall *et al.* (1963) also found that sheep erythrocytes were the most sensitive to lysis by alpha toxin. They suggested that sheep blood may be used to estimate the toxin-producing abilities of *Cl. welchii*.

In our study, horse, rabbit, and human erythrocytes were lysed by some strains which did not haemolyse sheep blood. Thus, it was difficult to determine whether the absence of haemolysis was due to the resistance of the erythrocytes to lysis, or to the differences in the amounts of alpha toxin produced. One strain, UM 37, did not produce alpha-toxin haemolysis on sheep blood, even though it produced high levels of alpha toxin (127·5 μg./ml.) as determined by the lecithovitellin method. Differential susceptibility of erythrocytes of different species to haemolysis may be more significant than the ability of a particular strain to produce alpha toxin.

The strains of *Cl. welchii* isolated from food were less haemolytic than the strains

isolated from faeces or soil. Of the strains isolated from foods, only 43 % produced haemolysis due to alpha toxin and 75 % produced haemolysis due to theta toxin. Sixty per cent of the strains isolated from soil and 61 % of the strains isolated from faeces produced alpha toxin haemolysis on all four bloods. Ninety-three per cent of the strains isolated from soil and 100 % of the strains isolated from faeces produced theta toxin haemolysis.

Table 2. *Haemolytic activity of* Clostridium welchii

Type of blood ...	Haemolysis due to theta toxin: Diameter (mm).*				Haemolysis due to alpha toxin: diameter (mm.)*			
	Horse	Sheep	Rabbit	Human	Horse	Sheep	Rabbit	Human
				Faeces				
UM 2	2·3	4·3	3·6	3·9	7·1	9·5	8·2	11·7
UM 3	2·5	3·2	8·5	1·9	—	9·6	—	—
UM 5	1·9	3·3	3·4	2·3	—	8·0	7·0	5·7
UM 6	2·4	3·4	6·8	2·6	—	8·1	—	7·9
UM 8	2·5	3·1	8·1	2·3	—	9·5	—	9·8
UM 9	1·7	2·6	3·1	2·1	—	7·3	8·7	8·3
UM 10	1·8	2·4	6·8	2·5	—	7·9	—	8·8
UM 12	1·9	2·9	7·6	2·6	—	9·7	—	—
UM 13	2·0	2·0	3·1	1·7	2·3	9·0	6·1	5·5
				Soil				
UM 31	2·4	3·3	8·7	2·9	—	9·8	—	—
UM 32	—	—	5·4	2·2	—	5·5	—	7·1
UM 33	2·7	3·3	9·0	2·7	—	8·3	9·7	9·9
UM 34	1·7	2·7	4·6	2·0	1·9	7·0	9·1	8·3
UM 35	2·5	3·8	4·9	3·2	—	8·5	8·7	7·9
UM 36	2·0	3·1	7·6	2·8	—	8·2	—	9·1
UM 37	—	6·5	5·3	7·2	—	—	5·6	—
UM 38	2·1	3·9	4·7	3·5	—	8·3	8·4	8·5
UM 39	1·9	2·9	8·4	2·4	—	9·2	12·3	10·9
UM 43	2·4	2·5	4·5	2·1	—	4·2	—	5·4
				Food				
UM 7	1·9	2·6	2·2	3·8	—	6·1	7·8	8·6
UM 17	1·5	2·8	6·9	2·3	—	5·5	—	—
UM 19	1·9	2·0	8·8	1·2	—	9·0	—	—
UM 26	2·6	4·0	8·7	3·2	—	9·7	—	10·7
UM 29	1·6	3·2	7·8	2·7	—	7·3	—	—
UM 46	—	4·3	4·0	1·9	—	—	—	5·5
UM 48	—	—	8·2	—	—	9·6	—	9·7
UM 52	—	—	5·7	—	—	7·6	—	8·6
UM 54	3·3	2·3	6·3	2·2	—	5·5	—	8·6
UM 55	—	—	8·1	—	—	9·5	—	8·5

* Mean of ten determinations. —, No haemolysis.

All three strains which produced heat-resistant spores were isolated from food. Two of these strains, UM 48 and UM 55, failed to produce theta toxin on horse, sheep, and human blood. These strains also produced low levels of lecithinase as determined by the lecithovitellin reaction. Therefore these strains fit Hobbs's criteria for food-poisoning strains. However, the majority of strains examined in

this investigation produced theta toxin, and therefore do not fit Hobbs's description of food-poisoning strains of *Cl. welchii*.

The haemolytic activity of the alpha and theta toxins was not strictly dependent on colony size. For example, two strains with identical colonial diameters produced areas of haemolysis varying from 23·8 mm.^{2} to 73·9 mm.^{2} on sheep blood plates.

The lecithinase activity of *Cl. welchii* did not correlate with the haemolytic activity for the majority of the strains. Some strains which produced large amounts of alpha toxin, as measured by the lecithovitellin technique, did not produce large areas of haemolysis. This relationship varied with the type of erythrocytes used to study the haemolytic activity.

Because the area of haemolysis due to theta toxin is considerably smaller than the area due to alpha toxin, it is possible that the theta toxin diffuses more slowly through the blood agar or that it undergoes rapid decomposition. The area of haemolysis due to alpha toxin on all bloods tested was subject to greater variation than the area of haemolysis produced by theta toxin. Of the twenty-three strains which produced both alpha and theta toxin on sheep blood, the standard deviation of the computed areas of theta toxin haemolysis was $\pm$ 2·8 mm.^{2}, whereas the standard deviation of alpha toxin haemolysis was $\pm$ 17·6 mm.^{2}. Quantitatively, the production of alpha toxin was much more variable than the amount of theta toxin produced.

Occasionally, the haemolytic patterns on various bloods deviated from the characteristic alpha toxin haemolysis or theta toxin haemolysis usually observed. The haemolytic variability among the strains and difference in the erythrocytes may have been responsible for these atypical zones of haemolysis. It is also possible that these haemolytic peculiarities were due to the unclassified non-alpha-delta-theta type of haemolysis described by Brooks *et al.* (1957) or the hypothetical X and Y haemolysis of van Heyningen (1941). Hall *et al.* (1963) also observed haemolysis produced by type A strains which was not due to alpha, theta, or delta toxins. Conceivably, some strains of *Cl. welchii* produce additional haemolysins which have not been identified.

Effect of temperature and length of incubation

The temperature and length of subsequent incubation similarly influenced both alpha and theta toxin activities. The additional incubation was conducted under aerobic conditions, therefore the additional haemolysis we noted was not due to the growth-stimulating effect of temperature. This treatment probably affected the red blood cells and previously synthesized toxins.

The haemolytic activity due to the theta toxin of *Cl. welchii* UM6 at different temperatures of subsequent incubation is presented in Fig. 1. The diameter of haemolysis was much greater on rabbit blood than on sheep, human or horse blood. On horse blood the haemolytic activity was highest at 52° C., whereas haemolysis was greatest at 37° C. on sheep and human blood. This suggests that the erythrocytes from different species may be reacting differently to the toxin. A number of complex interacting mechanisms may be involved. Higher temperatures may

cause an increased rate of diffusion of the toxin, but in some cases may cause a degradation of the toxin. In addition, higher temperatures may increase the fragility of erythrocytes, thus making them more susceptible to lysis.

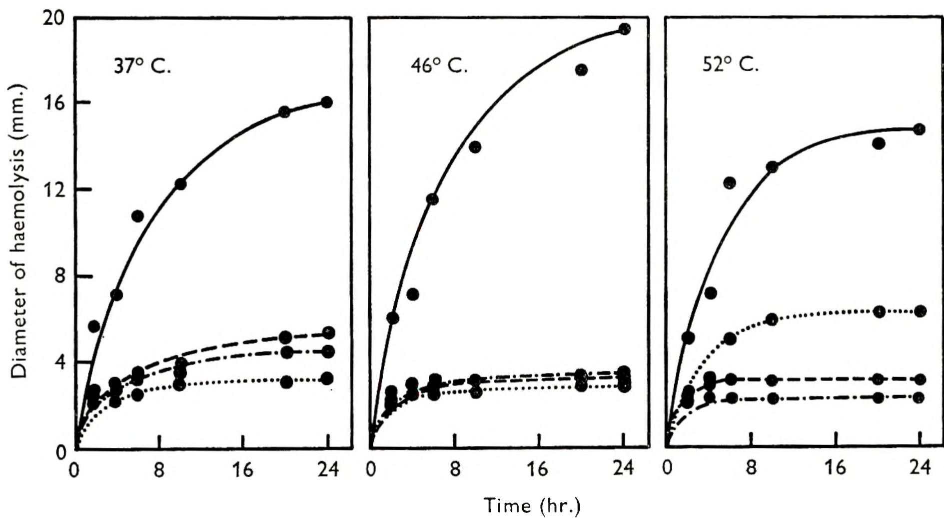


Fig. 1. Haemolytic activity of *Cl. welchii* UM 6 due to theta toxin on erythrocytes of different animal species as a result of additional incubation. —, Rabbit; – – –, sheep; —·—, human; ......, horse.

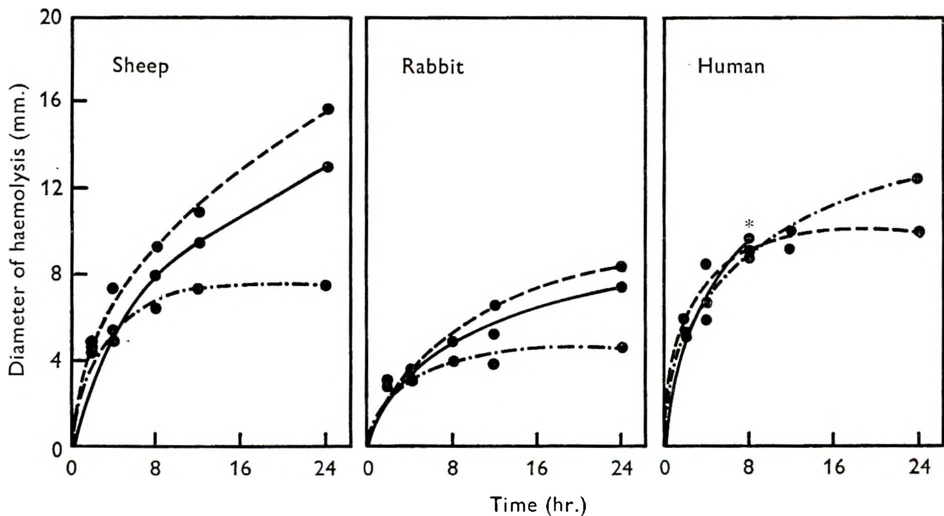


Fig. 2. Effect of time and temperature on the haemolytic activity of commercial lecithinase (80 μg./ml.) —, 37° C.; - – –, 46° C.; — · —, 52° C.;* haemolysis fades and was impossible to measure after 8 hr.

The diameter of haemolysis increased with increasing time of incubation. In studies dealing with the kinetics of haemolysins, Burrows (1951) reported that haemolysis due to alpha toxin of *Cl. welchii* was a function of time, while Bernheimer (1947) found a similar relationship with theta toxin.

Haemolytic activity of commercial lecithinase

To our knowlege the haemolytic activity of commercial lecithinase has not been related to that of alpha toxin from culture filtrates of *Cl. welchii*. The haemolytic activity of commercial lecithinase was directly proportional to the concentration and length of incubation. Haemolysis was produced by the enzyme on sheep, rabbit and human blood. Neither the commercial lecithinase nor the enzyme produced by the majority of the strains of *Cl. welchii* caused alpha toxin haemolysis on horse blood.

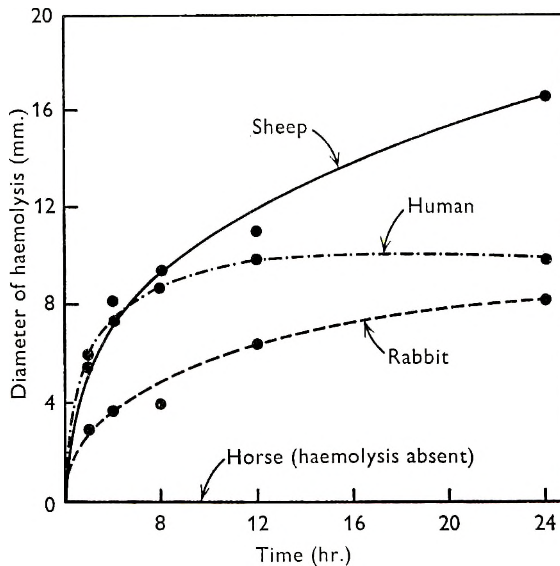


Fig. 3. Haemolytic activity of commercial lecithinase (80 μg./ml.) at 46° C. on erythrocytes of different animal species.

The effect of commercial lecithinase upon erythrocytes from different species is illustrated in Fig. 2. At a concentration of 80 μg./ml. greatest haemolytic activity was observed on sheep blood. Maximum haemolysis occurred when the blood plates containing the commercial lecithinase were incubated at 46° C. Clearly, there was a differential susceptibility of the different bloods to haemolysis by the commercial lecithinase (Fig. 3).

In Fig. 4 the haemolysis due to alpha toxin of *Cl. welchii* UM6 is compared to the haemolytic activity of commercial lecithinase. The concentration of lecithinase tested was 80 μg./ml. and the level of alpha toxin produced by UM6, as measured by the lecithovitellin technique, was also 80 μg./ml.

The close relationship between the haemolytic activity of *Cl. welchii* alpha toxin and commercial lecithinase indicates that these two have similar, if not identical, effects on red blood cells. These data confirm the hypothesis presented by Macfarlane & Knight (1941) that the alpha toxin of *Cl. welchii* is probably identical with lecithinase.

The haemolytic activity of commercial lecithinase was destroyed by exposure to 70° C. for 20 min. (Fig. 5). Partial inhibition of haemolytic activity occurred at

60° C. Little or no inhibition was observed when the enzyme was heated at 50° C. In studies from our laboratory (unpublished) it was found that lecithinase activity, as determined by the lecithovitellin technique, was almost completely destroyed when exposed for 5 min. at 60° and 90° C. Therefore the haemolytic activity of lecithinase appears to be more stable at 60° C. than the lecithovitellin activity of the same preparation. To our knowledge, this is the first report of the effects of heat upon the haemolytic activity of commercial lecithinase.

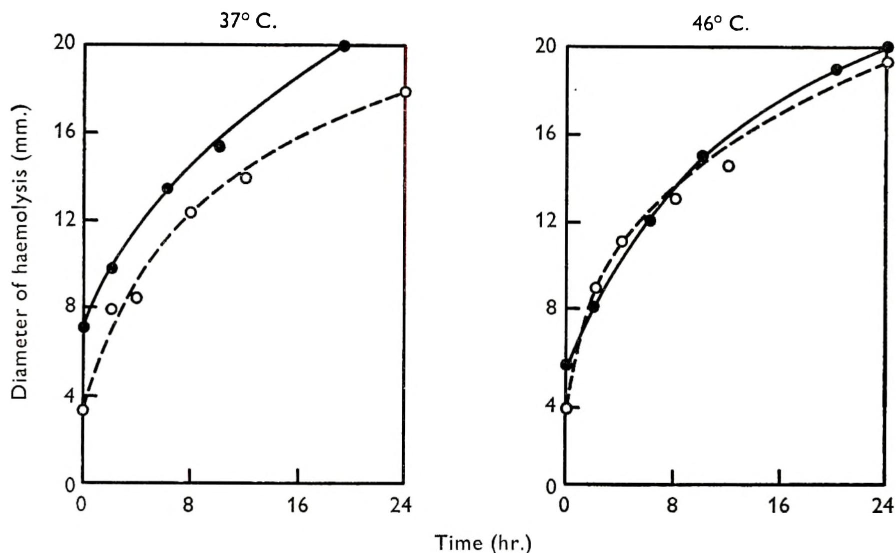


Fig. 4. Comparison of haemolysis due to alpha toxin of *Cl. welchii* UM 6 with haemolysis due to commercial lecithinase. ●—●, UM 6; ○—○, commercial lecithinase (80 μg./ml.).

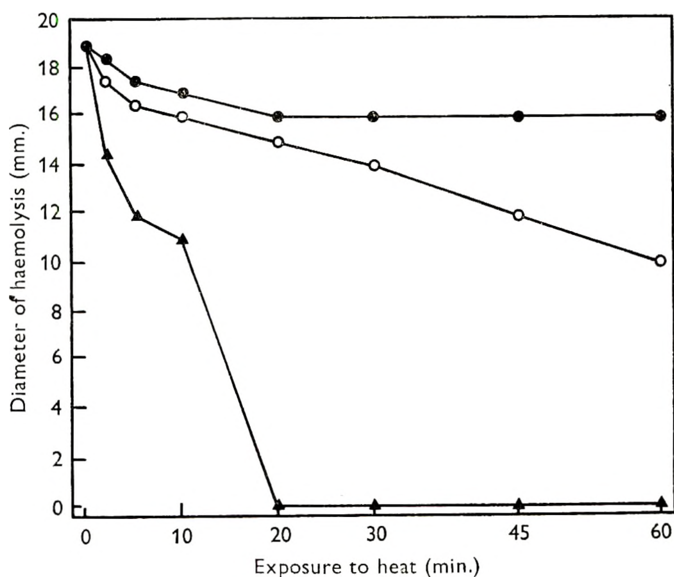


Fig. 5. Effect of heat on the haemolytic activity of commercial lecithinase. ●—●, 50° C.; ○—○, 60° C.; ▲—▲, 70° C.

DISCUSSION

It is apparent that a number of complex factors are involved in the haemolysis produced by the alpha and theta toxins of *Cl. welchii*. The variation in the differential haemolytic activity of *Cl. welchii* is consistent with other reports (Macfarlane, 1950; Hall & Hauser, 1966). Macfarlane, Oakley & Anderson (1941) concluded that differences in haemolysis by alpha toxin were due primarily to variations in the rate of hydrolysis of phospholipids in the red cells of different species. Obviously, other factors are also involved in differential haemolysis.

Stereochemical configuration is involved in the availability of specific combining sites on the enzyme. This may determine whether the active site of the lecithinase can affect the lecithins of the erythrocyte membrane. The variation in rate of enzyme action with erythrocytes from different species may be due to differences in the rate of absorption of the alpha toxin by the red cells. Variations in the fragility of red blood cells may also affect the degree of haemolysis.

Another factor to be considered is the variation in the dimensions of red cells from different species. The red cells of horse, sheep, rabbit and man are not only of different sizes, but also of different shapes (Ponder, 1948). The size and shape of the erythrocytes may be an important factor in the formation of enzyme-substrate complexes.

Among the various lipid components of red blood cells, the alpha toxin of *Cl. welchii* attacks lecithin, sphingomyelin, and phosphatidylethanolamine. There are only small differences in the amount of total phospholipids in red cells of horse, sheep, rabbit, and man (de Gier & van Deenen, 1961; Ponder, 1948). The differential susceptibility of different erythrocytes to haemolysis cannot be accounted for solely on the basis of the substrate present in the red cell membrane. Haemolytic differences may be due primarily to differences in the relative amounts of specific phospholipids present.

Although the nature of the theta toxin is not known, its mode of action resembles that of an enzyme. Our observations suggest that the mechanism of action of alpha and theta toxin upon blood may be similar. A majority of the strains which produced theta toxin haemolysis also produced alpha toxin haemolysis on the same blood. Temperature and length of subsequent incubation affected similarly the haemolytic activity of both toxins. A clear distinction between the two haemolytic zones was not always possible. When high concentrations of commercial lecithinase were tested, a clear zone of haemolysis within the zone of partial haemolysis was observed.

Gale & van Heyningen (1942) found that theta toxin was detectable in the growth medium a short time before the alpha toxin was detected. On blood plates the zone of alpha-toxin haemolysis surrounds the area of theta-toxin haemolysis. Therefore, it is plausible that the haemolytic activity of theta toxin occurs before that of alpha toxin.

On the basis of these observations, it is possible to hypothesize that one molecular species is responsible for both types of haemolysis. Different active groups present on the toxin moiety may be responsible for both partial and complete

haemolysis. Red blood cells are completely lysed under conditions of maximum enzymic activity. As the enzymic activity decreases, fewer erythrocytes may be acted upon, and partial haemolysis occurs. If only one molecular component is responsible for both types of haemolysis, the alpha-toxin haemolysis may follow theta-toxin haemolysis upon dissociation of the molecule.

SUMMARY

The lecithinase and haemolytic activity of thirty strains of *Cl. welchii* isolated from food, faeces, and soil, was studied. The strains from foods produced smaller amounts of lecithinase and were, in general, less haemolytic than the strains isolated from soil and faeces.

The haemolytic activity of *Cl. welchii* on erythrocytes from different animal species displayed considerable variation. Sheep erythrocytes were the most sensitive to the action of alpha toxin, whereas rabbit blood was most sensitive to haemolysis by theta toxin. The degree of haemolysis was also dependent upon the concentration of the enzyme, and temperature and length of incubation.

The haemolytic activity of commercial lecithinase was observed to be similar to the haemolytic activity of the alpha toxin of *Cl. welchii*. This finding provides further evidence that the haemolytic and lecithinase activities of *Cl. welchii* are due to one substance, the alpha toxin. Exposure of commercial lecithinase to heat resulted in the destruction of its haemolytic properties.

REFERENCES

Bernheimer, A. W. (1947). Comparative kinetics of hemolysis induced by bacterial and other hemolysins. *J. gen. Physiol.* **30**, 337.

Brooks, M. E., Sterne, M. & Warrack, G. H. (1957). A reassessment of the criteria used for type differentiation of *Clostridium perfringens*. *J. Path. Bact.* **74**, 185.

Burrows, W. (1951). Kinetic aspects of hemolysis by the α toxin of *Clostridium welchii* and its inhibition by antitoxin. *J. infect. Dis.* **89**, 233.

De Gier, J. & van Deenen, L. L. M. (1961). Some lipid characteristics of red cell membranes of various animal species. *Biochim. biophys. Acta* **49**, 286.

Dische, F. E. & Elek, S. D. (1957). Experimental food-poisoning by *Clostridium welchii*. *Lancet* ii, 71.

Gale, E. F. & van Heyningen, W. E. (1942). The effect of pH and the presence of glucose during growth on the production of α and θ toxins and hyaluronidase by *Clostridium welchii*. *Biochem. J.* **36**, 624.

Hall, H. E., Angelotti, R., Lewis, K. H. & Foter, M. J. (1963). Characteristics of *Clostridium perfringens* strains associated with food and food-borne diseases. *J. Bact.* **85**, 1094.

Hall, H. E. & Hauser, G. H. (1966). Examination of feces from food handlers for salmonellae, shigellae, enteropathogenic *Escherichia coli*, and *Clostridium perfringens*. *Appl. Microbiol.* **14**, 928.

Hauschild, A. H. W. & Thatcher, F. S. (1967). Experimental gas gangrene with food-poisoning *Clostridium perfringens* type A. *Can. J. Microbiol.* **14**, 705.

van Heyningen, W. E. (1941). The biochemistry of the gas gangrene toxins. I. Estimation of the α toxin of *Cl. welchii*, type A. *Biochem. J.* **35**, 1246.

Hobbs, B. C. (1965). *Clostridium welchii* as a food poisoning organism. *J. appl. Bact.* **28**, 74.

Hobbs, B. C., Smith, M. E., Oakley, C. L., Warrack, G. H. & Cruickshank, J. C. (1953). *Clostridium welchii* food poisoning. *J. Hyg., Camb.* **51**, 75.

Macfarlane, M. G. (1950). The biochemistry of bacterial toxins. 5. Variation in haemolytic activity of immunologically distinct lecithinases towards erythrocytes from different species. *Biochem. J.* **47**, 270.

MACFARLANE, M. G. & KNIGHT, B. C. J. G. (1941). The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. *Biochem. J.* **35**, 884.

MACFARLANE, M. G., OAKLEY, C. L. & ANDERSON, C. G. (1941). Haemolysis and the production of opalescence in serum and lecitho-vitellin by the α toxin of *Clostridium welchii*. *J. Path. Bact.* **52**, 99.

NAKAMURA, M. & CROSS, W. R. (1968). The lecithinase (α toxin) activity of strains of *Clostridium perfringens*. *Proc. Soc. exp. Biol. Med.* **127**, 719.

NEILL, J. M. (1926). Studies on the oxidation and reduction of immunological substances. II. The hemotoxin of the Welch Bacillus. *J. exp. Med.* **44**, 215.

PONDER, E. (1948). *Hemolysis and Related Phenomena*. New York: Grune and Stratton.

ROTH, F. B. & PILLEMER, L. (1955). Purification and some properties of *Clostridium welchii* type A theta toxin. *J. Immun.* **75**, 50.

SUTTON, R. G. A. & HOBBS, B. C. (1968). Food poisoning caused by heat-sensitive *Clostridium welchii*. A report of five recent outbreaks. *J. Hyg., Camb.* **66**, 135.

TODD, E. W. (1941). The oxygen-labile haemolysin or θ-toxin of *Clostridium welchii*. *Br. J. exp. Path.* **22**, 172.

The Journal of Hygiene Volume 67, No. 1 March 1969

CONTENTS

PAGE

BEARE, A. S., TYRRELL, D. A. J., HOBSON, D., HOWELLS, C. H. L., PEREIRA, M. S., POLLOCK, T. M. and TYLER, L. E. Live influenza B vaccine in volunteers . . . 1

SMORODINTSEV, A. A., DUBOV, A. V., ILYENKO, V. I. and PLATONOV, V. G. A new approach to development of live vaccine against tick-borne encephalitis . . . 13

HENDERSON, R. J. The outbreak of foot-and-mouth disease in Worcestershire. An epidemiological study: with special reference to spread of the disease by wind-carriage of the virus . . . 21

HARDING, LYNN and WILLIAMS, R. E. O. Selection of *Staphylococcus aureus* in cultures from air samples . . . 35

OXFORD, J. S. and POTTER, C. W. Chick embryo lethal orphan (CELO) virus as a possible contaminant of egg-grown virus vaccines . . . 41

CALIA, F. M., WOLINSKY, E., MORTIMER, E. A. JR, ABRAMS, J. S. and RAMMELKAMP, C. H. JR. Importance of the carrier state as a source of *Staphylococcus aureus* in wound sepsis . . . 49

AULICIEMS, A. Thermal requirements of secondary schoolchildren in winter . . . 59

POTTER, C. W. HI antibody to various influenza viruses and adenoviruses in individuals of blood groups A and O . . . 67

SANFORD, D. A., LESLIE, D. A., MCKEON, J. A., CRONE, P. B. and HOBBS, BETTY C. *Salmonella senftenberg* in the Sunderland area . . . 75

HOBBS, BETTY C. and HUGH-JONES, M. E. Epidemiological studies on *Salmonella senftenberg*. I. Relations between animal foodstuff, animal and human isolations . . . 81

HUGH-JONES, M. E. Epidemiological studies on *Salmonella senftenberg*. II. Infections in farm animals . . . 89

LIDWELL, O. M. and TOWERS, A. G. Protection from microbial contamination in a room ventilated by a uni-directional air flow . . . 95

STEELE, P. R. M., DAVIES, J. D. and GREAVES, R. I. N. Some factors affecting the viability of freeze-thawed T4 bacteriophage . . . 107

O'REILLY, K. J. and WHITAKER, A. M. The development of feline cell lines for the growth of feline infectious enteritis (panleucopaenia) virus . . . 115

PUBLIC HEALTH LABORATORY SERVICE. The occurrence of *Coxiella burnetii* in North-Western England and North Wales . . . 125

IVESON, J. B., MACKAY-SCOLLAY, E. M. and BAMFORD, V. *Salmonella* and Arizona in reptiles and man in Western Australia . . . 135

CUMMING, J. D. and MCEVEDY, C. P. An outbreak of 'winter vomiting disease' in a university hall of residence . . . 147

NAKAMURA, M., SCHULZE, JUDITH A. and CROSS, W. R. Factors affecting lecithinase activity and production in *Clostridium welchii* . . . 153

SCHULZE, JUDITH A. and NAKAMURA, M. Haemolytic activity of the alpha and theta toxins of *Clostridium welchii* . . . 163

Printed in Great Britain at the University Printing House, Cambridge