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Statistical analysis of staphylococcal infection in hospital patients in relation to use of antibiotics and other factors

By A. W. Voors and G. T. Stewart

Department of Epidemiology, School of Public Health, University of North Carolina, and the North Carolina Memorial Hospital

(*Received* 8 *September* 1967)

INTRODUCTION

The increasing importance of hospital cross-infection as a by-product of improved drug therapy entails the need for operational indices of the epidemicity of such infection. It is only by using such indices that institutional measures intended to decrease hospital infection can be evaluated objectively. Unfortunately, it is often impossible to conclude for a given strain of micro-organisms isolated from a hospital patient whether it is the result of: (*a*) infection of the patient before his admission, (*b*) activation of an organism which had colonized passively in the patient before his admission, or (*c*) actual infection in the hospital. However, it is generally suspected that many of the penicillin-resistant staphylococci isolated from hospital patients belong to the last category.

In this respect, the following finding is of interest. The coagulase or mannite positive staphylococci isolated from hospital patients can be plotted in a frequency curve according to the size of the growth-free zone in a disk test for sensitivity to penicillin-G. This curve is trimodal (Fig. 1),* implying that there are three populations of such staphylococci with distinctly differing degrees of resistance to penicillin-G: (1) the sensitive; (2) the moderately resistant; (3) the highly resistant (Lindberg & Stewart, 1965). Strains capable of producing penicillinase, spontaneously or inducibly, which are therefore advantageously placed when penicillin is being used, are found predominantly in the third category.

The objective of this study is to investigate whether this distinction offers an operational index of epidemicity of staphylococcal hospital infection which could be used in the evaluation of control programmes. This is done by seeing whether there is an ecological difference between staphylococci highly resistant to penicillin and those of moderate resistance. The ecological determinants under study are: (1) duration of hospital stay before the isolation of the organism; (2) use of penicillin-G during this stay; and (3) use of, combined with resistance to, other anti-

* It can be argued that the mode in the left of the figure is not obvious since it corresponds to a large interval on the measurement scale. Technical restrictions of the disk method used did not allow further specification of zone sizes smaller than a millimeter. However, even if this were possible and a frequency curve were plotted on a logarithmic scale allowing for a large number of intervals for this 'mode', the presence of at least one mode below size 10 could hardly be denied in view of the large total of such observations, practically irrespectively of the shape of the curve.

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biotics. If it transpires that one of the two penicillin-resistant groups is not introduced from outside the hospital and that antibiotic use has no effect upon the incidence of this group, then this will suggest hospital infection as the sole explanation of the existence of this resistance group, in which case the incidence of this group can be used as a specific index of hospital infection.

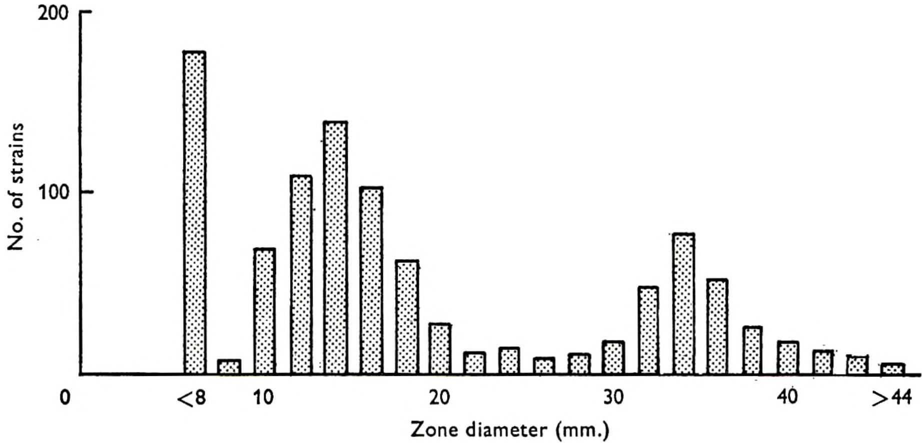

Fig. 1. Zones of inhibition produced in 1000 strains of *Staphylococcus aureus* by penicillin-G (10 units) disks. (Consecutive isolates in North Carolina Memorial Hospital during the period of surveillance.)

MATERIALS AND METHODS

From October 1964 until October 1965 all strains of coagulase or mannite positive staphylococci isolated in the North Carolina Memorial Hospital were referred from the diagnostic laboratory to the epidemiologic laboratory in the School of Public Health. Clinical data about the patient from whom each strain was isolated were obtained from the ward or case-chart. Each strain was examined for sensitivity to several antibiotics by the 10-unit disk method, and tested for penicillinase production by (*a*) a membrane-plate technique (Holt & Stewart, 1963), and (*b*) an indicator-dye method (Novick & Richmond, 1965). The characteristics for a given strain, including those of the patient from whom it was isolated, were punched in a card (Table 1). Further strains from this patient were similarly entered in a fresh card. A total of 2007 strains were thus documented in more or less detail. For each patient the clinical information was completed after the patient's discharge.

The data were examined with two questions in mind: (1) How often were patients admitted with staphylococci either moderately or highly resistant? (2) Could antibiotic use be excluded as a determinant of one of the two resistance groups of strains?

Table 1. *Partial code sheet, staphylococcal hospital infection study North Carolina Memorial Hospital*, 1964–5

Rank order of staphylococcus isolated
Patient identification number
Date of admission
Days after admission this organism isolated (= selected period)
Use of penicillin-G during selected period
Use of chloramphenicol during selected period
Use of tetracyclin during selected period
Use of kanamycin during selected period
Use of erythromycin during selected period
Zone size of disk test (penicillin-G)
Result of test for β-lactamase (membrane)
Result of test for β-lactamase (dye)
Sensitive or resistant to chloramphenicol
Sensitive or resistant to tetracycline
Sensitive or resistant to kanamycin
Sensitive or resistant to erythromycin

How often were patients admitted with staphylococci either moderately or highly resistant?

No systematic data were available concerning the strains from patients before admission. Data on staphylococcal infection in the Intensive and Special Care Service of North Carolina Memorial Hospital collected from August 1964 through April 1965 give the impression that the median time interval between infection and detection as practised here is not longer than 6 days (Voors, 1967*a*). Hence the strains isolated from patients 6 days or less after their admission were analysed separately from those from patients having stayed 7 days or more. Thus, the proportion of all strains that is highly resistant was calculated for each duration-of-stay group, and the same was done for the moderately resistant strains. Here the strains at risk of becoming or being moderately resistant are limited to the sensitive and moderately resistant groups, and do not include the highly resistant according to current bacteriological theory. Hence, here the moderately resistant proportion of the sum of moderately resistant and sensitive strains was calculated for each duration of stay before isolation of these strains. The resulting proportions are called 'proportion of strains highly resistant' and 'proportion of strains moderately resistant' respectively. These four proportions were compared by chi-square technique. The legitimacy of combining the various durations of stay within the four groups was assessed by analysis of variance.

Could antibiotic use be excluded as a determinant of either moderately or highly resistant strains?

The number of highly resistant strains was compared with the sum of the moderately resistant and sensitive strains for various combinations of characteristics measured. Likewise, the number of moderately resistant strains was compared with the number of penicillin sensitive strains. These data were tested for independence by chi-square technique.

RESULTS

How often were patients admitted with staphylococci either moderately or highly resistant?

The number of strains is tabulated by degree of penicillin resistance and by duration of prior stay in Table 2.

Table 2. *Staphylococcal infections in hospital. Strains by degree of resistance to penicillin-G and by duration of stay prior to isolation*

Duration of prior stay (days)	Degree of resistance of strain			
	High	Moderate	None	Total
0	65	298	221	584
1	15	34	37	86
2	3	18	17	38
3	0	23	9	32
4	5	3	9	27
5	8	14	10	32
6	0	9	1	10
7	3	12	2	17
8	5	11	0	16
9	5	15	3	23
10–19	57	73	23	153
20–29	18	39	7	64
30–39	17	25	2	44
40–49	14	1	3	18
50–59	7	1	3	11
60–69	1	9	3	13
70–79	0	3	1	4
80–89	0	0	0	0
90+	2	5	0	7
Total	225	603	351	1179

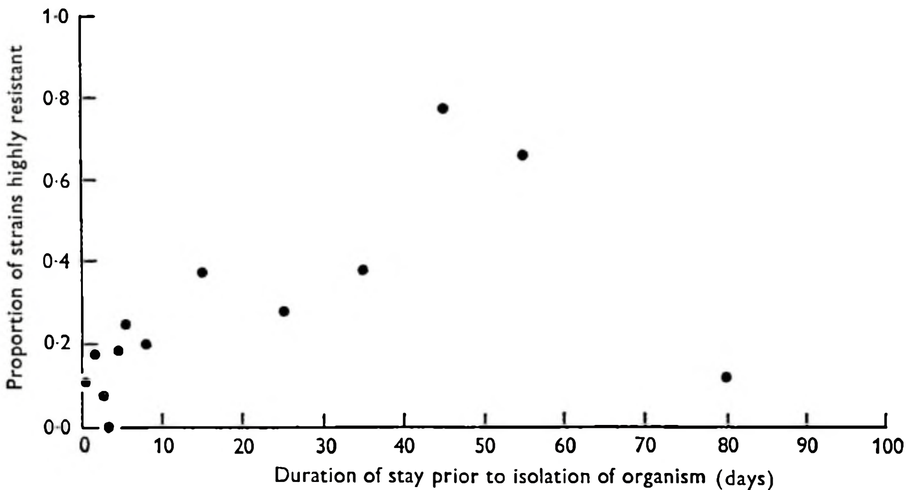

Fig. 2. Proportion of staphylococcal strains highly resistant to penicillin-G, by duration of prior stay in hospital.

The proportion of strains highly resistant is plotted by duration of prior stay in Fig. 2 and of strains moderately resistant in Fig. 3. Each of these two groups of proportions was divided into a category with stay durations of 6 days or less and one of 7 days or more.

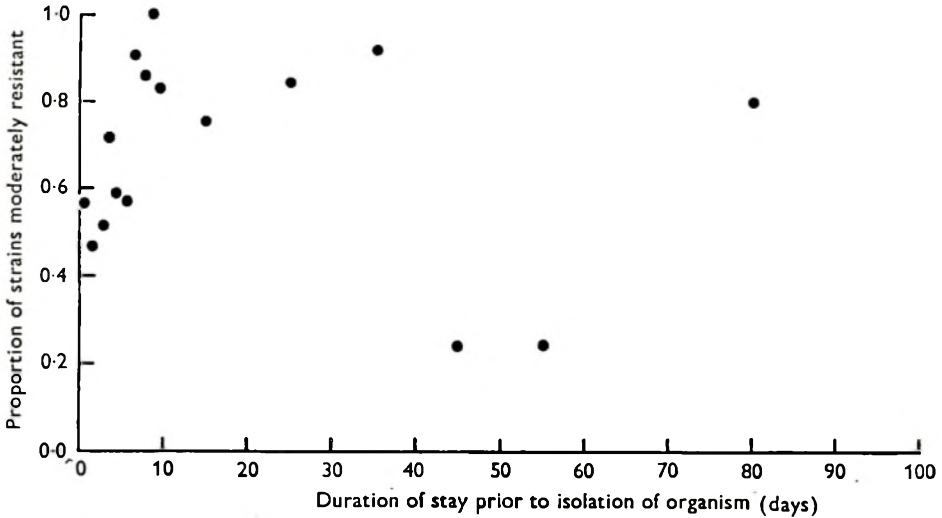

Fig. 3. Proportion of staphylococcal strains moderately resistant to penicillin-G, by duration of prior stay in hospital.

Table 3. *Staphylococcal infections in hospital. Strains by degree of resistance to penicillin-G, by use of penicillin-G, and by duration of prior hospital stay*

Days of prior hospital stay	Use of Penicillin-G	Resistance to penicillin-G			
		High	Moderate	None	Total
0–5	+	30	167	118	315
	–	37	126	111	274
6–19	+	43	69	10	122
	–	27	50	19	96
20+	+	32	48	7	87
	–	26	34	12	72
Total	+	105	284	135	524
	–	90	210	142	442

When a weighted regression of the proportions against the duration of stay was performed, the weight being inversely proportional to the estimated variance [i.e. if the proportion is $p_i = r_i/n_i$ for any duration-of-stay i, then the weight is

$$w_i = \frac{n_i^3}{r_i(n_i-r_i)} = \frac{n_i}{p_i(1-p_i)}\Bigg],$$

it was found in all four groups analysed that neither the linear nor the quadratic term was significant at the 5% level when tested against the residual from a quadratic trend. Thus, the data are not inconsistent with the contention that after the sixth day of admission the proportions remain fairly constant.

By chi-square technique it was found that the short-stay long-stay ratio of proportions highly resistant was very significantly smaller than that of proportions moderately resistant. The data (Fig. 2) suggest that few if any of the highly resistant strains were acquired before admission.

Could antibiotic use be excluded as a determinant of either moderately or highly resistant strains?

The number of strains by degree of resistance, duration of prior stay and use of penicillin-G during this stay are given in Table 3.

Table 4. *Strains by degree of resistance to penicillin-G, by duration of prior hospital stay, and by use* of chloramphenicol*

Days of prior hospital stay	Use of* chloram-phenicol	Resistance to penicillin-G			
		High	Moderate	None	Total
0–19	+	23	21	0	44
	–	114	391	258	763
20+	+	19	13	2	34
	–	39	69	17	125
Total	+	42	34	2	78
	–	153	460	275	888

* Combined with resistance to chloramphenicol.

Table 5. *Strains by degree of resistance to penicillin-G, by duration of prior hospital stay, and by use* of tetracycline*

Days of prior hospital stay	Use of tetra-cycline	Resistance to penicillin-G			
		High	Moderate	None	Total
0–19	+	24	40	0	64
	–	113	372	258	743
20+	+	19	32	1	52
	–	39	50	18	107
Total	+	43	72	1	116
	–	152	422	276	850

* Combined with resistance to tetracycline.

Application of the chi-square test for independence to this table as described above indicates that, with regard to the number of strains, the relative frequency of high resistance is not significantly dependent upon use of penicillin-G in these patients at the 5% level of significance. However, the effect of moderate resistance is dependent on the effect of use of penicillin-G at the 5% level of significance.

The numbers of strains by degree of resistance, duration of prior stay and use of four other common antibiotics (chloramphenicol, tetracycline, kanamycin and erythromycin) are given in Tables 4–7. Application of the chi-square test in the same manner as described above results in rejection of the null-hypothesis at the 5% level of significance for all four antibiotics and both resistance groups.

Table 6. *Strains by degree of resistance to penicillin-G, by duration of prior hospital stay, and by use* of kanamycin*

Days of prior hospital stay	Use of* kana-mycin	Resistance to penicillin-G			
		High	Moderate	None	Total
0–19	+	8	5	0	13
	–	129	407	258	794
20+	+	5	12	0	17
	–	53	70	19	142
Total	+	13	17	0	30
	–	182	477	277	936

* Combined with resistance to kanamycin.

Table 7. *Strains by degree of resistance to penicillin-G, by duration of prior hospital stay, and by use* of erythromycin*

Days of prior hospital stay	Use of* erythro-mycin	Resistance to penicillin-G			
		High	Moderate	None	Total
0–19	+	5	7	0	12
	–	132	405	258	795
20+	+	12	11	0	23
	–	46	71	19	136
Total	+	17	18	0	35
	–	178	476	277	931

* Combined with resistance to erythromycin.

INTERPRETATION OF RESULTS

There is a prevailing impression that staphylococci with high resistance to penicillin-G belong to the hospital environment and are seldom introduced from outside. The data here support this impression.

The next question is whether the acquisition of such staphylococcal strains is influenced by their production of penicillinase when penicillin is given to a patient who might have been infected with a predominantly sensitive strain before his admission, or is due to infection within the hospital. An attempt to answer this question is made by assessing the association between use of antibiotics and degree of resistance to penicillin-G.

If attention is focused on strains of staphylococci with high resistance to penicillin-G, the present study suggests that use of this drug is not associated with an excessive isolation rate of these strains over other strains of lesser resistance. This is not true, however, for the use of four other antibiotics (chloramphenicol, tetracyclines, kanamycin and erythromycin); isolation of strains highly resistant to penicillin-G is associated with the use of any one of the four other antibiotics just mentioned. Hence we may speculate that use of penicillin-G does not favour the acquisition (by its user individually) of staphylococci highly resistant to this drug, although since the organisms concerned were, for the most part, shown in

the study to be producers of penicillinase, the biologic effect of use over a period must have constituted a positive selective pressure favouring persistence of these strains in the hospital environment: use of other antibiotics, however, was found to be associated with penicillin resistance as indicated by an excessive rate of highly penicillin-resistant organisms isolated from the users of these antibiotics. It is therefore possible that this use of non-penicillin drugs is actually favouring penicillin resistance.

If attention is focused on strains of staphylococci with moderate resistance to penicillin-G, both use of this drug and of the four other antibiotics is significantly associated with this resistance. In the case of penicillin-G, this association is stronger here than in the highly resistant strains.

These findings suggest that the highly penicillin-resistant staphylococci in hospital patients, who did not receive other antibiotics to which these strains are also resistant, are rarely introduced into the hospital by a newly admitted patient and are rarely induced in a patient by his use of penicillin-G in the circumstances of the present study. Hence they must be largely due to infection after admission, or related to use of antibiotics other than penicillin-G.

IMPLICATION

Under the conditions as inferred above, the threshold theorem (Bailey, 1957) predicts that, as soon as, for a ward or service, the product of rate of contact between individuals and expected duration of infectivity after infection decreases below the value one (the 'epidemic threshold'), the incidence of the relevant strains will virtually drop to zero. The model on which the threshold theorem is based is not invalidated by abandoning the assumption of closed communities (Voors, 1967*b*). Therefore, the proportion of highly penicillin-resistant staphylococci from patients who did not receive broad-spectrum antibiotics could be used as an index of success in a control programme of hospital infection.

Thus, there may be practical merit in routinely distinguishing between the three degrees of resistance to penicillin-G in staphylococci. The two resistant groups can be easily separated by their position on the zone-frequency curve (Fig. 1). In view of the observed association it would be desirable in this respect to disregard the strains also resistant to other antibiotics and isolated from patients who used these antibiotics. However, it is proposed that, if the laboratory facilities are limited, the index of hospital infection be restricted to those highly penicillin-resistant strains isolated from patients who did not receive other antibiotics during their hospital stay. If fuller laboratory facilities are available, the epidemiologic consequences of using antibiotics can be additionally assessed from the shape of the trimodal curve and, especially, from changes in population with moderate or intermediate resistance to penicillin G.

SUMMARY

When staphylococcal strains from hospital patients are plotted in a frequency curve according to the size of the growth-free zone in a disk test for sensitivity to penicillin-G, this curve is trimodal. This implies that, besides the sensitive, there are two populations of resistant staphylococci: the moderately and the highly resistant, In a hospital-wide survey of coagulase or mannite positive staphylococci isolated in North Carolina Memorial Hospital between October 1964 and October 1965 it was inferred that: (1) there was a statistically highly significant difference between the distributions of the moderately and the highly resistant strains over duration of hospital stay prior to the time of isolation; (2) the highly resistant strains were seldom introduced from outside; and (3) there was no significant association between use of penicillin-G by a patient harbouring a strain and the frequency with which such a strain was found to be highly resistant to this drug. The latter finding is in contrast to significant associations found when the penicillin resistance is moderate, or when the drug used is other than penicillin-G. It is concluded that a fall in the incidence of staphylococcal strains highly resistant to penicillin-G in patients who did not receive other antibiotics may well be a valid index of the efficacy of programmes to control hospital infection. If so, there is a practical merit in adopting the routine of distinguishing between two groups of penicillin-resistant staphylococci, which behave, epidemiologically, as two distinct bacterial populations.

Grateful acknowledgement is made to our colleagues in the clinical and microbiological departments of the North Carolina Memorial Hospital, in particular to Dr W. J. Cromartie, Dr Janet Fischer, Miss Charlotte Merrit, Mrs Betsy Sutton and Mr Brian Butcher. The University Computer (IBM 360) was used in the preliminary data analysis.

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Antibody to *Mycoplasma pneumoniae* in normal subjects and in patients with chronic bronchitis

BY H. P. LAMBERT
St George's Hospital, London, S.W. 17

(*Received* 4 *October* 1967)

Mycoplasma pneumoniae, formerly known as Eaton agent, causes respiratory illness which varies greatly in severity and type, and, much less commonly, a variety of non-respiratory clinical syndromes (Lambert, to be published). Infection may also be clinically silent, and serological surveys have shown that infection is common in many parts of the world (Hayflick & Chanock, 1965).

The sera of 1150 people mainly domiciled in the south of England were tested for antibody against *M. pneumoniae*. The sera of 140 people with chronic bronchitis were also examined in the same way.

METHODS

Complement-fixing antibody was measured by a standard macro-method, using 2 units of complement, 4 units of antigen and over-night fixation at 4° C. The antigen was at first prepared as described by Chanock *et al.* (1962). In this method, phenol is added to the crude culture grown in liquid medium as a means of decreasing the anticomplementary effect of the preparation. We obtained more satisfactory reduction of anticomplementary effect by washing the cultures, grown to counts of 10^6–10^7 viable units per ml., three times in veronal buffer, and then placing the final suspension in a boiling-water bath for 10 min. Satisfactory antigen was prepared by this method, but batches varied somewhat in titre and specificity. The problem of preparing a constant specific antigen of high titre was solved by the technique developed by Kenny & Grayston (1965). In this method the washed concentrated liquid culture is extracted by chloroform and methanol and then partitioned against 0·1 M–KCl. The final product is a soluble lipid antigen which is specific and of high titre. Antigen titres of 320, using extracts representing 100-fold concentration of the original culture, were commonly achieved. The latter part of the work was done with these extracted antigens and most of the earlier sera were also retested using these antigens. Sera were tested at a dilution of 1/10 and positive sera were titrated in a second test.

Series A consists of 623 sera sent to St George's Hospital Medical School for routine Kahn testing of applicants for visas to the U.S. Embassy. The range was wide, with a predominance of young people, and the only data available were age and sex. These sera may well have included some from people with chronic or recent acute chest illness. Series B consists of 527 unselected sera from in-patients of St George's Hospital whose blood was grouped for any reason. Full clinical

details were available and patients with chronic or recent acute chest illness were excluded. Series C consists of 140 sera from patients with chronic bronchitis attending St George's Hospital, Brompton Hospital, or chest clinics in the London area. All had been diagnosed by chest physicians as suffering from chronic bronchitis, and none of them had experienced a recent acute chest illness.

RESULTS

The proportion of positive sera was similar in series A and B (Table 1), and there was no significant difference between males and females in the prevalence of antibody to *M. pneumoniae* (Table 2). The pooled results of the two series of normal sera show an over-all prevalence of antibody of 18·6 %, rising to a peak of 27 % in the fourth decade, and then declining slowly to 16·4 % in all patients over 40, and 15·5 % in all patients over 50.

Table 1. Mycoplasma pneumoniae *antibody in normal subjects*

	Series A		Series B		Total A and B		
Age (years)	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive	% positive
0–9	—	—	47	5	47	5	10·6
10–19	32	5	27	4	59	9	15·3
20–29	194	30	54	15	248	45	18·1
30–39	160	47	70	15	230	62	27·0
40–49	104	18	100	19	204	37	18·1
50–59	73	13	84	13	157	26	16·6
60 and over	60	9	145	21	205	30	14·6
Total	623	122	527	92	1150	214	18·6

Table 2. Mycoplasma pneumoniae *antibody in normal subjects*

	Male			Female		
Age in (years)	No. tested	No. positive	% positive	No. tested	No. positive	% positive
0–19	45	5	11	61	9	15
20–29	89	17	19	159	28	18
30–39	102	25	24	128	37	29
40–49	71	14	20	133	23	17
50–59	68	11	16	89	15	17
60 and over	84	15	18	121	15	12
Total	459	87	19	691	127	18·4

Of the 140 patients with chronic bronchitis in series C, 50 (36 %) showed antibody to *M. pneumoniae* (Table 3). Their age distribution was, of course, different from that of the normal controls. Table 4 shows a matched group of patients over 40, including 137 of those with bronchitis, and 566 controls. Antibody to *M. pneumoniae* is much more commonly found in patients with chronic bronchitis than in normal people ($P < 0{\cdot}0001$).

Table 3. Mycoplasma pneumoniae *antibody in chronic bronchitis*

Age (years)	No. tested	No. positive	% positive
20–39	3	2	—
40–49	19	3	—
50+	118	45	38
Total	140	50	36

Table 4. Mycoplasma pneumoniae *antibody in subjects over* 40

	Normal		Chronic bronchitis	
	No. tested	% positive	No. tested	% positive
Male	233	18	115	32
Female	343	15	22	50
Total	566	16·4	137	35·0

DISCUSSION

Infection by *M. pneumoniae* is evidently common in southern England. The age distribution of antibody in normal subjects conforms with that of clinical infection by this organism, which is common in children (but not infants) and young adults and less common in the middle-aged and elderly.

The greater than normal incidence of antibody to *M. pneumoniae* in patients with bronchitis suggests that their undue tendency to acquire respiratory infections includes a susceptibility to this particular pathogen. The range of antibody titres found (mostly 40 or less) in bronchitis was, however, no different from those found in sera from normal people. Since complement-fixing antibody tends to decline or disappear in time, the findings suggest that *M. pneumoniae* infection remains more common or more persistent in patients with bronchitis at an age when it has become an uncommon infection in normal people. Thus, one-third of patients with bronchitis had antibody and they were nearly all over 40, whereas only one-sixth of normal people over 40 showed antibody to *M. pneumoniae* in their serum.

The relatively transient nature of complement-fixing antibody proved an advantage in demonstrating this tendency of bronchitic patients to go on acquiring *M. pneumoniae* infection into middle and old age. For the same reason, the results using the complement-fixation method must underestimate the total number of past *M. pneumoniae* infections in the normal population tested. Other more recently developed methods of antibody measurement may prove more suitable than the complement-fixation test in future epidemiological studies. Of these, the tetrazolium reduction test (Taylor-Robinson, Sobeslavsky, Jensen, Senterfit & Chanock 1966) appears to be specific and sensitive. The indirect haemagglutination-inhibition test, utilizing sensitized tanned red cells, is sensitive but suffers from the disadvantage of poor specificity. For example, 60 % of sera from children 2–4 years old gave positive results in 1/10 dilution, a result inconsistent with the known

epidemiology of *M. pneumoniae* infection (Taylor-Robinson, Shirai, Sobeslavsky & Chanock, 1966).

The percentage of positive results found at different ages by these two methods suggests that antibody persists for many years; and antibody measured by another new technique, that of haemagglutination inhibition (Feldman & Suhs, 1966), is known to persist for at least 10 years. The complement-fixation method is, however, a satisfactory one for routine work in the diagnosis of Eaton agent infection, especially if a soluble extracted antigen is used (Kenny & Grayston, 1965).

Patients with chronic bronchitis seem to be unduly susceptible to a variety of respiratory pathogens, and the agents associated with exacerbations are those prevalent in the community. For example, Ross *et al.* (1966) found rising antibody titres to influenza A and to respiratory syncytial virus in bronchitic exacerbations when these agents were prevalent, and Somerville (1963) reported an association between respiratory syncytial virus infection and exacerbations of chronic bronchitis at a time when this virus was prevalent in the community. In normal adults respiratory syncytial virus causes no symptoms or minor respiratory illness.

Vaccines against *M. pneumoniae* have already been prepared and tested (Smith, Friedewald & Chanock, 1967; Metzgar *et al.* 1966). They may prove valuable in military and other establishments in which Eaton agent infection is prevalent, but infection in the general civilian population is not common or severe enough to justify their large-scale use. If a prospective study confirms that patients with chronic bronchitis suffer unduly from *M. pneumoniae* infections, vaccination against this organism may have to be added to the protective measures taken for patients with chronic respiratory disease.

SUMMARY

Mycoplasma pneumoniae is a common respiratory pathogen in the south of England. Of 1150 sera from normal people, 19% had antibody against this organism. Antibody was found with significantly greater frequency in patients with chronic bronchitis.

It is a pleasure to thank those who have given me help: Drs J. Angel, J. Batten, and P. Zorab for many sera from bronchitic patients, Professor S. D. Elek for many helpful discussions, and Miss M. Knobel for technical assistance. The work was supported by grants from the Medical Research Council and by the Board of Governors of St George's Hospital.

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The inhibition of growth of vaccinia and cowpox viruses in RK 13 cells

BY DERRICK BAXBY AND C. J. M. RONDLE*
Department of Bacteriology, Liverpool University, Liverpool 3, *England*

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INTRODUCTION

Many workers have studied the inhibition of growth of vaccinia virus. They have used various virus strains, cell lines and experimental conditions.

Early work (Thompson, 1947) was concerned primarily with the effect of inhibitors on the production of infective virus; later work concerned the effect of growth inhibitors on the production of viral antigens. Using complement fixation, immune fluorescence, and precipitation of radio-active proteins 'vaccinia-specific antigen' has been detected in cultures treated with sodium azide (Easterbrook, 1961), isatin-β-thiosemicarbazone (IBT; Easterbrook, 1962), 5-fluorodeoxyuridine (FUDR; Salzman, Shatkin & Sebring, 1963), 5-bromodeoxyuridine (BUDR; Easterbrook & Davern, 1963) and hydroxyurea (Rosenkranz, Rose, Morgan & Hsu, 1966).

Gel-diffusion techniques have shown the complexity of pox virus-specific antigens (Gispen, 1955; Rondle & Dumbell, 1962; Appleyard & Westwood, 1964; Marquardt, Holme & Lycke, 1965). Such techniques have shown also that the production of some rabbit pox soluble antigens is inhibited by azide and IBT (Appleyard, Westwood & Zwartouw, 1962), by rutilantin A (Hume, Westwood & Appleyard, 1965), but not by BUDR (Appleyard & Westwood, 1964).

Attempts have been made to correlate the varied data available for pox viruses, although Joklik (1966) has emphasized the dangers of comparing results obtained in different ways. This paper compares the effect of several growth inhibitors on the replication of one strain each of vaccinia and cowpox. A standard set of experimental conditions was adopted, and wherever possible one cell line (RK 13) was used; exceptions are noted in the text. The criteria of growth studied were infective virus production and the appearance of virus specific soluble antigens as detected by gel diffusion and haemagglutination tests.

MATERIALS AND METHODS

Virus strains

The Lister strain of vaccinia and the Brighton strain of cowpox were used throughout.

* Present address: London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1.

Cell lines

The RK 13 line of transformed rabbit kidney cells (Beale, Christofinis & Furminger 1963) was used for most of the experiments; in some cases HeLa cells (strain Conway) were used.

Cells were grown as monolayers in 2 in. diam. Petri dishes in Parker's 199 medium. For RK 13 cells 5 % calf serum was added, for HeLa cells 5 % inactivated human serum. Incubation was at 35° C. in an atmosphere of 5 % CO_2 in air. Eagle's basal medium was used without serum for maintaining confluent monolayers (Baxby & Rondle, 1967).

Growth inhibitors

These are listed under their most probable mode of action as adjudged from the literature. Most of the compounds have been tested on poxvirus by several workers. Iodacetate was included as it has been overlooked since the work on vaccinia by Thompson (1947). Proflavine was tested because adenovirus replication was inhibited, although adenovirus components were synthesized in its presence (Wilcox & Ginsberg, 1962). Unless otherwise stated, inhibitors were stored at −20° C. as 0·1 M solutions in sterile 0·85 % saline.

Inhibitors of energy-yielding reactions: sodium azide (B.D.H.), sodium iodoacetate (B.D.H.).

Inhibitors of DNA function. Proflavine hydrochloride (K & K Labs.) and actinomycin D (a gift from Mercke, Sharpe & Dohme) which interfere with transcription of RNA from DNA.

Inhibitors of DNA synthesis: FUDR (a gift from Roche Products), BUDR (California Corp. for Biochem. Research), hydroxyurea (Sigma Chemical Co.), mitomycin C (Kyowa Hakko Kogyo Co.): this last compound was kept in the dark in the original ampoules and dissolved in maintenance medium immediately before use.

Inhibitors with specific activity against poxviruses: *n*-methyl-IBT (MIBT) (a gift from Dr D. J. Bauer, Wellcome Labs.). This was dissolved at 2 mg./ml. in dimethylformamide and diluted to 100 μM. in water. The aqueous suspension was autoclaved at 15 lb./in.^{2} for 10 min. to dissolve the compound. Solutions were stored at 37° C. and diluted with maintenance medium when required. Rutilantin A (a gift from Dr G. Appleyard, M.R.E. Porton) was dissolved in acetone at 200 μg./ml., stored at −20° C. and diluted with maintenance medium when required.

Experiments with proflavine, actinomycin D and mitomycin C were done in subdued light.

Antisera

Antisera to vaccinia and cowpox were prepared in rabbits as described by Rondle & Dumbell (1962). Rabbit-grown virus only was used for immunization to avoid production of antibodies not specific for virus.

Virus growth curves

Growth curves were done as described by Baxby & Rondle (1965). Virus multiplicities of 10 and 0·01 plaque-forming units (pl.f.u.) per cell were used with three

dishes per group. Inhibitors were usually added with the inoculum. Cells and supernatant fluids were harvested separately at various intervals after inoculation. Cells were disrupted ultrasonically (M.S.E./Mullard disintegrator) and the virus content of disrupted cells and supernatant fluid determined.

Virus infectivity titrations

These were done either on the chorioallantoic membrane (CAM) of 12-day chick embryos (McCarthy & Dumbell, 1961) or by the RK 13 plaque assay (Baxby & Rondle, 1967).

Plaque inhibition tests

Confluent RK 13 monolayers were inoculated with approx. 240 pl.f.u. virus in 1 ml. medium containing inhibitor. After 1 hr. at 35° C. 3 ml. medium containing inhibitor was added and incubation continued. Plaques were counted and measured at the optimum time. This was 46 hr. after inoculation for vaccinia and 50 hr. after inoculation for cowpox (Baxby & Rondle, 1967). The percentage plaque reduction compared with controls was calculated.

Haemagglutination tests

These were done as described by McCarthy & Helbert (1960).

Gel diffusion tests

The Ouchterlony double-diffusion technique was used as modified by Rondle & Dumbell (1962). Fluids and cells from infected cultures were harvested separately, dialysed at 4° C. against daily changes of distilled water until free from salt, and dried from the frozen state. Dried materials were resuspended at suitable concentrations in phosphate-phosphate buffer, pH 7·4 for test. Results of experiments were photographed by dark ground illumination.

Virus antigens

Vaccinia standard antigen (VA) was made from infected rabbit dermis. Cowpox standard antigen (CA) was obtained from infected RK 13 cells.

RESULTS

VIRUS REPLICATION IN RK 13 CELLS

Virus growth

Growth curves of vaccinia and cowpox in RK 13 cells using an input multiplicity of 10 pl.f.u./cell are shown in Fig. 1. Fifty per cent of the virus was adsorbed to the cells by 1 hr. (Table 1). Figure 1 shows that new infective virus was detected at 8–10 hr. for vaccinia and 10–12 hr. for cowpox; and that maximum titres were detected at 20 hr. for vaccinia and at 30 hr. for cowpox.

Even by 48 hr. little virus had been released from the cells into the supernatant fluids. The virus yields for a range of inocula are shown in Table 1. At the end of one growth cycle there was a yield of approximately 100 pl.f.u./pl.f.u. adsorbed

for both viruses. With inocula above 1×10^7 pl.f.u./dish (5 pl.f.u./cell) the yield remained constant at 48 hr. By 48 hr. with lower inocula second cycles of infection gave a higher yield than at 24 hr.

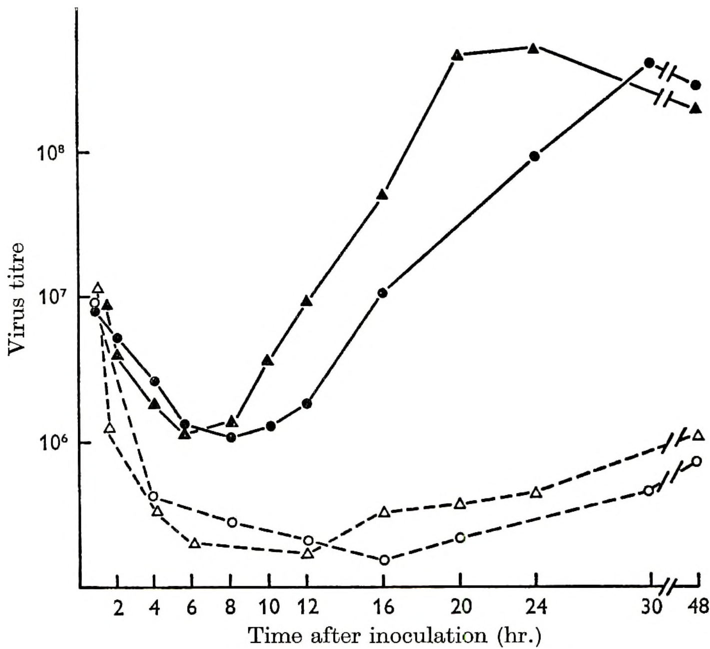

Fig. 1. Growth curves of vaccinia and cowpox viruses in RK 13 cells. ▲——▲, Cell-associated vaccinia virus; △----△, fluid-associated vaccinia virus; ●——●, cell-associated cowpox virus; ○----○, fluid-associated cowpox virus.

Table 1. *Yield of vaccinia and cowpox in RK 13 cells*

Inoculum (pl.f.u./dish 10^5)	Virus adsorbed* at 1 hr. (10^6)	Virus yield (10^6)	Virus yield/virus adsorbed, $\log_{10}$	Virus yield (10^7)	Virus yield/virus adsorbed, $\log_{10}$
		24 hr.		48 hr.	
A. Vaccinia					
0·1	0·05	0·5	2·0	41	4·9
2·0	1·1	6·2	1·8	32	3·5
10	4·7	67	2·1	36	2·8
100	50	320	1·8	42	1·9
200	91	500	1·8	47	1·7
		30 hr.		48 hr.	
B. Cowpox					
0·1	0·06	0·65	2·1	30	4·7
2·0	1·2	10	1·9	37	3·5
10	6·1	52	1·9	41	2·8
100	51	300	1·8	35	1·9
200	100	590	1·8	53	1·7

* Virus adsorbed at 1 hr. calculated from inoculum virus titre less virus removed in the supernatant fluids at 1 hr.

Virus soluble antigens

Haemagglutinin (HA)

This was detected first at the time new infective virus was produced. Figure 2 shows the relationship between the yields of HA and infective virus at the end of one growth cycle. The particular strain of cowpox used produced HA as readily as vaccinia in RK13 cells. Fenner (1958) obtained the same result on CAM, although other strains of cowpox produced less HA than vaccinia. The mean value for the ratio virus yield (pl.f.u./ml.)/HA (units/ml.) was $10^{5.0}$ for both viruses. At the end of one growth cycle the HA titres in the cells were at least 30 times higher than the titres in the overlaying fluids.

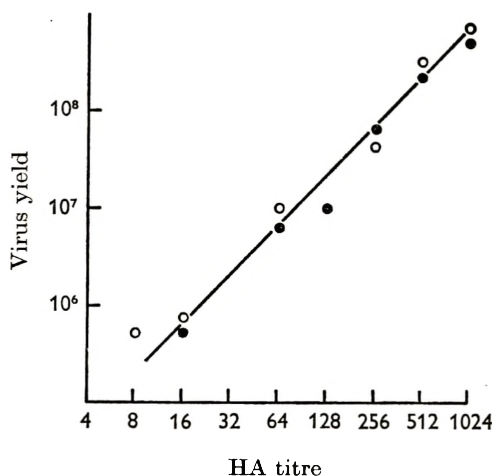

Fig. 2. Relationship between yield of infective virus and yield of HA at the end of one growth cycle. ●, Vaccinia virus; ○ cowpox virus.

Antigens detected by gel diffusion

Plates 1A and B show the 'standard system' used for the analysis of vaccinia and cowpox soluble antigens. By suitable tests thirteen different lines were regularly detected in gel diffusion patterns. Numbers were allotted to the lines and used to designate the antigens or haptens which were line pattern components (lpc). Numbers 1 and 2 do not appear in this paper; they were used in the course of the work to designate line pattern components which were not detected regularly. Lpc 3, 5 and 7 were not normally found in cowpox soluble antigens prepared from CAM (cf. lpc *f* in Rondle & Dumbell, 1962). Lpc 3 is heat labile and lpc 5 and 7 heat stable; they are serologically related to the 'L' and 'S' antigens of vaccinia (Smadel & Shedlovsky, 1942; Williamson, 1963; Williamson & Rondle, 1964). Lpc *d* was the material normally present in cowpox soluble antigens, but absent from antigens prepared from vaccinia and the white pock variant of cowpox (Rondle & Dumbell, 1962).

Plate 2A shows that all the components present in *VA* were present in vaccinia soluble antigens produced in RK13 cells with the possible exception of lpc 1 and 2, which were not regularly detected in standard antigen *VA*. Plate 2A also shows that

the water-insoluble buffer-soluble fraction (*VARK bs*) deposited during dialysis contains a full complement of lpc, whereas the water-soluble fraction (*VARK ws*) contained only lpc 10, 11 and 12 with traces of lpc 8 and 9. Dialysis thus presented a convenient method of concentrating vaccinia soluble antigens. Cowpox soluble antigens shared many components with *VA* and *VARK*, but lpc *d* was not present in the vaccinia-infected tissues and lpc 11 was never demonstrated in cowpox soluble antigens. Although cowpox soluble antigens from CAM lack lpc 3, 5 and 7 (Plate 1A) cowpox soluble antigens from RK13 cells contained lpc 3 and small amounts of lpc 5, 7 (Plate 1B). Rondle & Dumbell (1962) extracted lpc 3 and 5 from cowpox-infected CAM with trypsin, and it is possible that these components may be present in a diffusible form in cowpox-infected RK13 cells.

The distribution of lpc between cells and culture fluids was determined. All components were detected in cells, but lpc 3, 4, 5, 6, 7 and 11 were not detected in culture fluids. This may be a general feature of poxvirus-infected tissue cultures since similar results were obtained with chick embryo fibroblasts, HeLa and GMK cells. The qualitative difference in lpc distribution between cells and fluids permitted recognition of two further components, lpc 13 and 14, which were readily detected in the fluids but obscured in tests on cell extracts by other components (Plate 2B).

Tables 4 and 5 (controls) show the times after inoculation at which each lpc could be detected in infected RK 13 cells. The common components appeared approximately 2 hr. later in cowpox-infected RK13 cells than in vaccinia-infected RK13 cells. Lpc *d* was the first component detected in cowpox-infected tissues.

Events following infection of RK13 cells treated with inhibitors of virus growth

Virus production

Sodium azide

This compound was cytotoxic, but comparison of infected and uninfected monolayers in the presence of inhibitor suggested that virus-specific cytopathic effect occurred in cultures treated with 2·5 mM. but not 5 mM. azide. No evidence of infective virus synthesis was obtained from growth curves done in the presence of the latter concentration. Results obtained with vaccinia and cowpox were identical and similar to those described for rabbit pox in HeLa cells (Appleyard *et al.* 1962) and for vaccinia in KB cells (Easterbrook, 1961).

Sodium iodoacetate

This compound was cytotoxic and plaque inhibition tests could not be done. However, growth curves showed that cowpox virus replication was completely inhibited by 50 μM. iodoacetate (Fig. 3B), although this concentration permitted approximately 5 % vaccinia virus synthesis (Fig. 3A). Such a difference in the effect of iodoacetate on closely related viruses has not previously been reported.

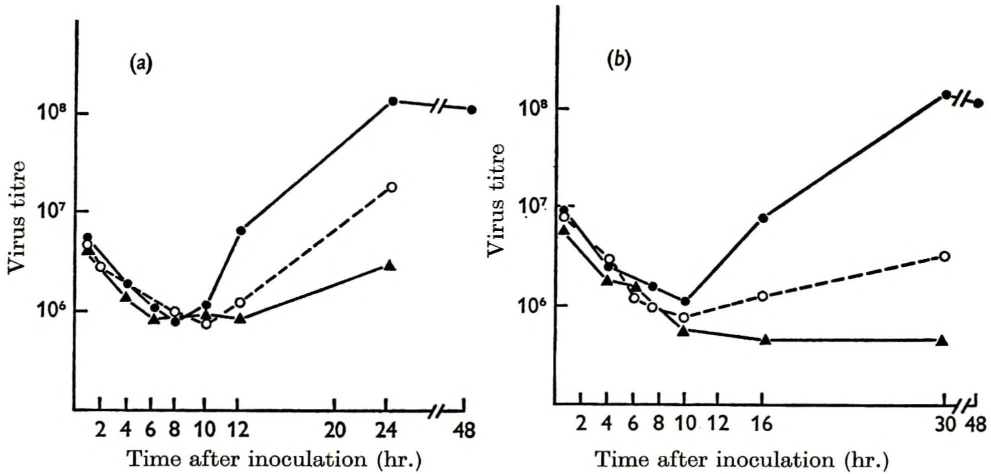

Fig. 3. Effect of iodoacetate on growth curves of (*a*) vaccinia and (*b*) cowpox in RK 13 cells. ●——●, Control; ○----○, 20μM.; ▲——▲, 50μM. iodoacetate.

Proflavine and actinomycin D

Plaque inhibition tests and growth curve experiments showed that replication of vaccinia and cowpox viruses in RK 13 cells was completely inhibited by 20 μM. proflavine. A similar result was obtained for vaccinia in KB cells by Bubel & Wolff (1965). Replication of both viruses was completely inhibited by 0·5 μg/ml. actinomycin D.

FUDR

It is difficult to inhibit poxvirus replication completely in RK 13 monolayers with FUDR (Baxby & Rondle, 1965). However, results of plaque inhibition and growth curve experiments indicated that vaccinia and cowpox virus replication was inhibited completely by 80 μM. FUDR.

BUDR

Replication of both viruses was completely inhibited by 25 μM. BUDR. This was of interest since Easterbrook & Davern (1963) showed that different poxviruses may differ in susceptibility to this reagent.

Hydroxyurea

Cowpox virus was completely inhibited by 10 mM. hydroxyurea (Fig. 4B), whereas a 50 mM. concentration was required to inhibit vaccinia (Fig. 4A). The result with cowpox was similar to that obtained with vaccinia in human amnion cells by Rosenkranz *et al.* (1966), but as far as is known the results reported here represent the first finding that this compound has different effects on closely related viruses.

Mitomycin C

Both viruses were completely inhibited in RK 13 cells by 10 μg./ml. mitomycin C. This compares well with the result obtained for vaccinia in HeLa cells by Oda (1963).

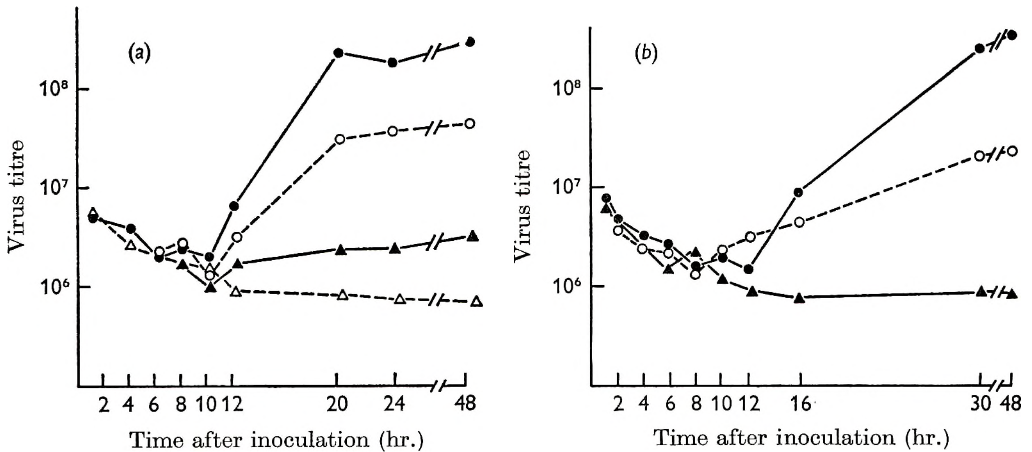

Fig. 4. Effect of hydroxyurea on growth curves of (*a*) vaccinia and (*b*) cowpox in RK 13 cells. ●——●, Control; ○----○, 1 mM; ▲——▲, 10 mM; △----△, 50 mM. hydroxyurea

Table 2. *Plaque inhibition tests with MIBT in HeLa cells*

MIBT (μM.) ...	0	10	20	30	40
Vaccinia count	100*	16	0	0	NT†
Plaque diam. (mm.)	0·7	0·1	0	0	NT†
Cowpox count	100*	36	9	1·5	0
Plaque diam. (mm.)	0·6	0·4	0·2	0·1	0

* Control counts adjusted to 100: other counts expressed as % of this.
† Not tested.

Thiosemicarbazone

Thiosemicarbazones are relatively inefficient in RK 13 cells (Appleyard, Hume & Westwood, 1965; Baxby, 1967). Experiments were done therefore in HeLa cells. Using these cells cowpox proved to be less susceptible to IBT than vaccinia (Table 2.). This finding corresponds to that of Bauer (1961) who studied the effect of IBT on the growth of these viruses in mice.

Rutilantin A

The minimum inhibitory concentration of this compound for both cowpox and vaccinia in RK 13 cells was 0·6 μg./ml. Hume *et al.* (1965) obtained a similar result for rabbit pox virus in these cells.

Haemagglutinin

Production of vaccinia and cowpox HA was inhibited by azide, iodoacetate, proflavine, actinomycin D, hydroxyurea, FUDR, MIBT and rutilantin A. Concen-

trations of these inhibitors which completely inhibited virus replication completely inhibited HA production. In cultures where virus replication was not inhibited completely the amount of HA produced was proportional to the virus yield. Vaccinia virus HA has been reported previously to be sensitive to inhibition in tissue culture by actinomycin D (Fujio, 1963), FUDR (Loh & Payne, 1965) and proflavine (Bubel & Wolff, 1965). Results with the other compounds are novel.

Oda (1963) reported that vaccinia virus HA was produced in HeLa cells in concentrations of mitomycin C (MC) which completely inhibited virus replication. We obtained a similar result using RK 13 cells (Table 3). No virus was produced at inhibitor concentrations of 10 μg/ml. and above, although significant amounts of HA were produced in cowpox infections at 15 μg./ml. and in vaccinia infections at 30 μg./ml. inhibitor. It is interesting that increasing concentrations of inhibitor affected cowpox HA more than vaccinia HA.

Table 3. *Effect of mitomycin C on HA production*

Mitomycin C (μg./ml.) ...	0	5	10*	15	20	25	30
Vaccinia HA	1024†	1024	1024	1024	512	64	16
Cowpox HA	1024	1024	1024	128	4	4	2
No virus	4	—	—	4	—	—	4

* No new virus produced with 10 μg./ml. mitomycin C or above.
† Figures are reciprocals of HA endpoint.

Vaccinia and cowpox HA were also produced in cultures treated with sufficient BUDR to inhibit virus replication completely. At BUDR concentrations of 25 μM. or above no new virus was produced. However, HA production by infected cells occurred even in the presence of 80 μM. BUDR. The titre of HA detected (1/1024) did not differ from that of controls with no inhibitor added.

Antigens detected by gel diffusion

The effects of inhibitors on the production of vaccinia and cowpox soluble antigens are shown in Tables 4 and 5. Results with both viruses are similar. Compounds which probably disrupted energy utilization in host cells (azide, iodoacetate) severely curtailed the production of detectable soluble antigens. This is despite the finding that new vaccinia virus was produced in the presence of 50 μM. iodoacetate. Compounds which inhibit DNA function (proflavine, actinomycin D) had a similar effect, although results obtained with actinomycin D depended upon amount of inhibitor used and time of application to the test system. Probable inhibitors of DNA synthesis (FUDR, BUDR, HU and MC) allowed production of most of the virus-specific substances found in infected RK 13 cells, although no new virus was produced. Indeed with BUDR and MC virus-specific HA was detected.

MIBT could not be used in RK 13 cells and experiments were done in HeLa cells. At the concentrations used the result obtained was similar to that given by MC in RK 13 cells except that HA was not produced. With rutilantin A a dose-response effect was found similar to that found with actinomycin D.

Table 4. *Effects of inhibitors on production of vaccinia soluble antigens in RK 13 cells*

Inhibitor	Components detected after inoculation at (hr.)					
	4	5	6	8–10	10	12
None (control)	10, 12	8, 9	6	4, 3, 5, 7	HA	14*, 11
Components detected 24 hr. after inoculation						
Azide, 5 mM.	10, 12	8, 9	—	—	—	—
Iodoacetate, 50 μM.†	10, 12	8, 9	—	—	—	—
Proflavine, 20 μM.	10, 12	—	—	—	—	—
Actinomycin D, 3 μg./ml.	10, 12	—	—	—	—	—
Actinomycin D, 0·5–2 μg./ml.	10, 12	—	—	—	—	14
Actinomycin D, 2 μg./ml.‡	10, 12	—	—	4	—	14
Actinomycin D, 0·5–1·5 μg./ml.‡	10, 12	8, 9	6	4	—	14
FUDR, 80 μM.	10, 12	8, 9	6	4, 3, 5, 7	—	14, 11
BUDR, 25–80 μM.	10, 12	8, 9	6	4, 3, 5, 7	HA	14, 11
Hydroxyurea, 50 mM.	10, 12	8, 9	6	4, 3, 5, 7	—	14, 11
Mitomycin C, 10–20 μg./ml.	10, 12	8, 9	6	4	HA	14, 11
MIBT, 20 μM.§	10, 12	8, 9	6	4	—	14
Rutilantin A, 1·2 μg./ml.	10, 12	—	—	—	—	14
Rutilantin A, 1·0 μg./ml.	10, 12	—	—	4	—	14
Rutilantin A, 0·6–0·8 μg./ml.	10, 12	8, 9	6	4	—	14

* Lpc 14 obscured in cells by other materials. Recognized in fluids at 12 hr.
† Virus synthesis detected.
‡ Inhibitor added 1 hr. after inoculation.
§ Experiments done in HeLa cells.

Table 5. *Effect of inhibitors on production of cowpox soluble antigens in RK 13 cells*

Inhibitor	Components detected after inoculation at (hr.)					
	4	6	8	10–12	12	16
None (control)	*d*	10, 12	8, 9, 6	4, 3, 5, 7	HA	13*, 14*
Components detected 30 hr. after infection						
Azide, 5 mM.	*d*	10, 12	8, 9	—	—	—
Iodoacetate, 50 μM.†	*d*	10, 12	8, 9	—	—	—
Proflavine, 20 μM.	*d*	10, 12	—	—	—	—
Actinomycin D, 3 μg./ml.	—	10, 12	—	—	—	13
Actinomycin D, 2 μg./ml.	*d*	10, 12	—	—	—	13, 14
Actinomycin D, 2 μg./ml.‡	*d*	10, 12	—	4	—	13, 14
Actinomycin D, 1·5 μg./ml.‡	*d*	10, 12	8, 9, 6	4	—	13, 14
FUDR, 80 μM.	*d*	10, 12	8, 9, 6	4, 3, 5, 7	—	13, 14
BUDR, 25–80 μM.	*d*	10, 12	8, 9, 6	4, 3, 5, 7	HA	13, 14
Hydroxyurea, 10 mM.	*d*	10, 12	8, 9, 6	4, 3, 5, 7	—	13, 14
Mitomycin C, 10–20 μg./ml.	*d*	10, 12	8, 9, 6	4	HA	13, 14
MIBT, 40 μM.§	*d*	10, 12	8, 9, 6	4	—	13, 14
Rutilantin A, 1·5 μg./ml.	*d*	10, 12	—	4	—	13, 14
Rutilantin A, 1·2 μg./ml.	*d*	10, 12	8, 9, 6	4	—	13, 14

* Lpc 13 and 14 obscured in cells by other lpc; detected in fluids at 16 hr.
† No virus synthesis detected.
‡ Inhibitor added 1 hr. after inoculation.
§ Experiments done in HeLa cells.

DISCUSSION

The results showed that in RK13 cells new virus was detected 8–10 hr. after inoculation with vaccinia and 10–12 hr. after inoculation with cowpox. Virus-specific soluble antigens were detected 4 hr. after inoculation with both viruses. Antigens shared by the viruses however were detected earlier in vaccinia-infected tissues than in cowpox-infected tissues. Baxby & Rondle (1967) observed that cowpox plaques developed more slowly than vaccinia plaques in RK13 cells, but reasons for this phenomenon are not known.

In this work thirteen line pattern components were regularly detected in gel diffusion tests. Ten were shared by both viruses but lpc 11 was found only in vaccinia-infected tissues and lpc *d* and 13 in cowpox-infected tissues. The result with lpc *d* agrees with the observations of Rondle & Dumbell (1962) using antigens prepared from infected CAM: the other results are novel and require further investigation to establish whether or not lpc 11 and 13 are respectively vaccinia and cowpox-specific.

The times at which each lpc was first detected are given in Tables 4 and 5. These might also indicate the order in which virus-specific materials were made. Detection of materials however depends on the antibody composition of the antiserum used and the concentration of antigen or hapten present in the preparation. Identical results were obtained using several antisera. Order of manufacture could be confused however if an 'early' antigen was produced so slowly that its detection was delayed and a 'late' antigen was produced so fast that it was detected quickly.

Survey of the effect of inhibitors of virus growth showed that compounds of similar probable activity had similar effects upon the replication of both viruses. Concentrations of inhibitor which prevented production of infective virus did not necessarily inhibit production of soluble antigens. Compounds which appear to interfere with DNA function had the most dramatic effect. Lpc 10 and 12 were the only virus-specific materials found in vaccinia-infected cells treated with proflavine. In cowpox-infected cells lpc *d* was detected in addition to lpc 10 and 12. With actinomycin D additional materials were detected (lpc 14 with vaccinia; lpc 13 and 14 with cowpox). Lpc 10, 12 and *d* were the first virus-specific materials detected in infected cultures not treated with inhibitors. It is possible that in cultures treated with inhibitors the materials found were breakdown products of the infecting virus. This is, however, unlikely as low virus doses were used and few specific materials were detected and it seems reasonable to suppose that virus breakdown would furnish more than two or three of the thirteen soluble antigens known.

The dose-response effect with actinomycin D suggests that the steps involved in virus replication are not uniformly sensitive to inhibition. The effect of delaying application of this inhibitor until 1 hr. after inoculation was also of interest. At 2 μg./ml. one additional virus-specific substance was detected (lpc 4); with lower concentrations of inhibitor a further three substances were found (lpc 8, 9 and 6). This suggests that pathways leading to the production of nine or more line pattern components (lpc 10, 12, 4, 8, 9, 6, 14, and in addition in cowpox lpc 13 and *d*) were

established quickly after infection. After 1 hr. these pathways were not susceptible to blockage by an inhibitor of RNA synthesis. Since not all the pathways required for virus synthesis were present it suggests further that manufacture of virus-specific material is a sequential process.

It is tempting to suggest that some of the materials produced early in the replication cycle might be enzymes necessary for producing virus structural components and DNA rather than being virus structural components themselves. New enzymes are known to be produced in pox virus-infected cells (reviewed by Joklik, 1966) and the 'new' DNA polymerase found in herpes-infected cells has virus specificity (Keir *et al.* 1966).

Inhibitors of energy-yielding reactions also had a marked effect on the production of pox virus soluble antigens. As with proflavine lpc 10 and 12 were detected in vaccinia-infected cells, and lpc *d*, 10 and 12 in cowpox-infected cells. In cultures infected with either virus, however, lpc 13 and 14 could not be found, although lpc 8 and 9 were regularly present. The materials synthesized were those recognized early in the time study of antigen production. This supports the view that virus synthesis in infected RK13 cells treated with azide or iodoacetate proceeds sequentially until energy supplies are exhausted.

The effects of inhibitors of DNA synthesis are of interest because inhibitor-treated infected cells produced most, if not all the virus-specific substances found in uninhibited infected RK13 cells. Moreover, three different results were obtained with the four inhibitors tested suggesting that different methods of inhibition were involved. It was not possible to demonstrate cessation of DNA synthesis in treated RK13 cells, but provided this was achieved the results showed that in the presence of BUDR, inoculum virus DNA could code for all the virus-specific materials, detected by gel diffusion and haemagglutination, normally associated with virus replication. In the presence of FUDR and HU the only virus-specific substance not detected was HA. With mitomycin C, HA was produced, but lpc 3, 5 and 7 were not detected in cowpox-infected cells, nor lpc 3, 5, 7 and 11 in vaccinia-infected cells. Failure of inoculum virus DNA to code for virus-specific material could be understood if the DNA were degraded. It is known that in other systems mitomycin C both inhibits DNA synthesis and potentiates degradation of DNA by DNA-ase (Kersten, 1962; Kersten *et al.* 1964; Pricer & Weissbach, 1964). If MC did not immediately affect the input DNA it would accord with the finding that it inhibited only the manufacture of the materials detected late in the time-study of antigen production.

Results with specific inhibitors of poxvirus growth require little comment apart from the relative inefficiency of thiosemicarbazones in RK13 cells. Rutilantin A showed a dose-response effect and results obtained with the smallest dose were identical with the results given by MIBT in HeLa cells.

Consideration of Tables 4 and 5 *in toto* shows that the suggested sequential synthesis of virus-specific materials cannot be simple. For example, lpc 4 was on occasion detected in inhibitor-treated, infected RK13 cells in the absence of lpc 8 and 9; it was never detected in the absence of lpc 10, 12 and 14. In other experiments lpc 8 and 9 were found in the absence of both lpc 4 and 14, and lpc 14 was

found in the absence of lpc 8, 9 and 4. A possible interpretation of these results is that following synthesis of lpc 10 and 12 one pathway leads to production of lpc 8 and 9 and another independent pathway to production of lpc 14 followed by lpc 4.

Similar reasoning suggests that lpc 6 is formed next, followed by lpc 3, 5 and 7. The materials which are possibly specific to vaccinia or cowpox, lpc *d*, 11 and 13, have been omitted from this sequence. It can be seen that the possible order of sequential synthesis deduced from studies with inhibitors is similar to the results of the time-study of antigen production. For complete agreement it is necessary only to postulate that lpc 4 is produced before lpc 6 and that lpc 14 is produced in the cells 7–9 hr. before its detection in the supernatant fluids.

The work described emphasizes the close relationship between vaccinia and cowpox viruses. New differences have been detected however; these are the possible qualitative differences in soluble antigens discussed previously, and the quantitative differences in the presence of various inhibitors: thus vaccinia virus production was less sensitive than cowpox to iodoacetate and hydroxyurea, and production of vaccinia HA was affected less by MC than was production of cowpox HA. On the other hand, cowpox virus production was less sensitive than vaccinia virus to MIBT and more rutilantin A was required to inhibit production of cowpox antigens than was required to inhibit the serologically identical materials in vaccinia soluble antigen.

It is not known if these results apply to other strains of these viruses as Easterbrook & Davern (1963) showed that different strains of vaccinia had different susceptibilities to BUDR. It is intended to compare the properties of other strains of vaccinia and cowpox viruses with those reported here for the Lister and Brighton strains.

SUMMARY

The growth of vaccinia and cowpox in RK 13 cells was studied by measurement of infective virus production, gel diffusion and haemagglutination. The effects of virus growth inhibitors on the normal course of infection were then followed. Although the results with the two viruses were essentially similar some differences were detected between them. Inhibitors of DNA synthesis permitted production of many virus-specific soluble antigens. Compounds which inhibit DNA function and compounds that affected energy-yielding reactions had more dramatic effects. The different results obtained suggested that the synthesis of virus-specific materials was sequential and a possible part of the sequence is suggested.

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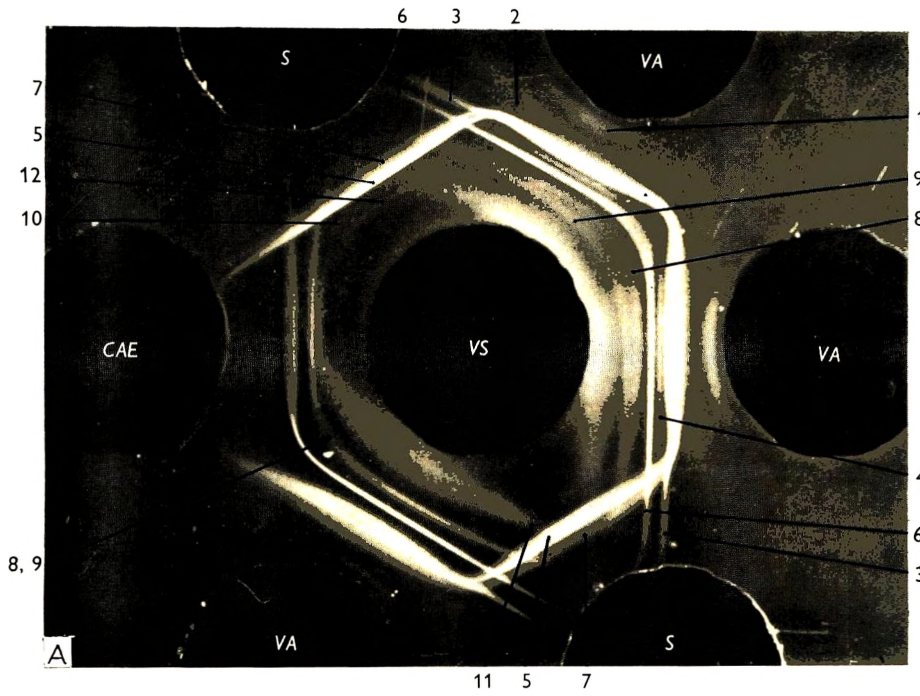
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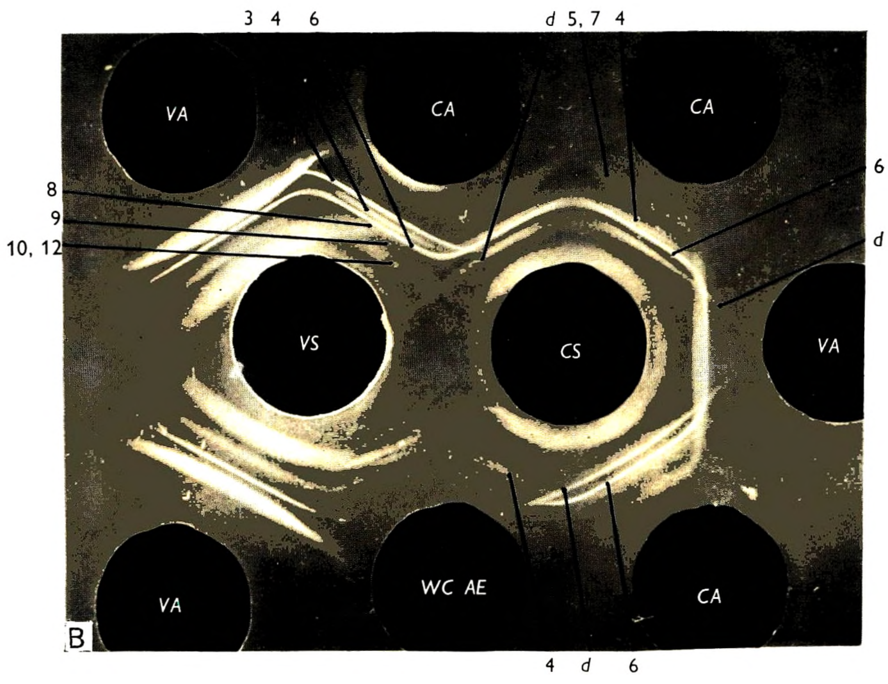
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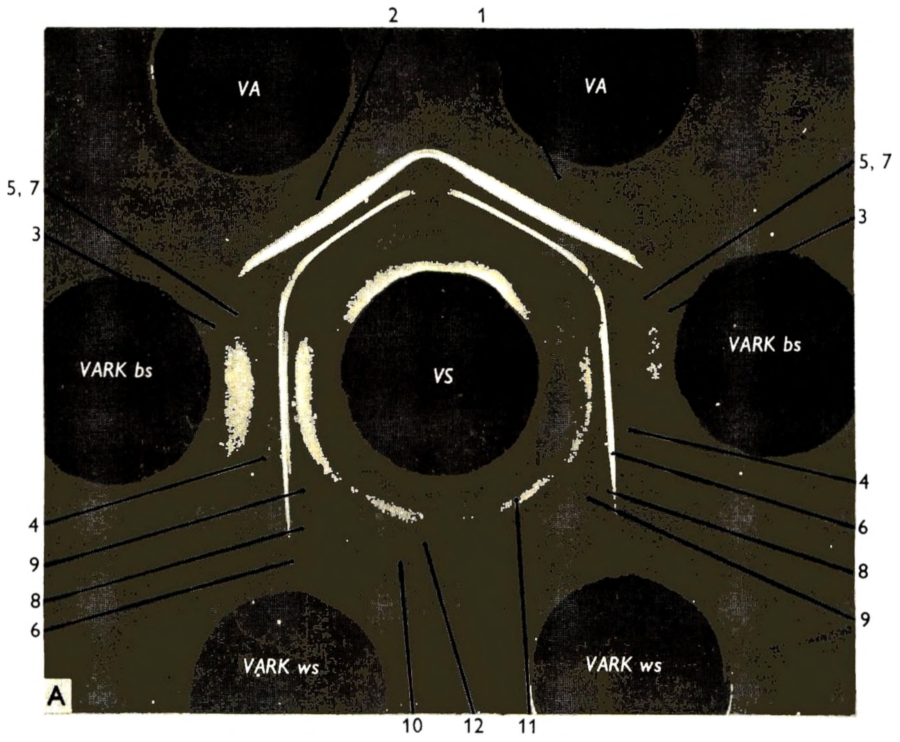
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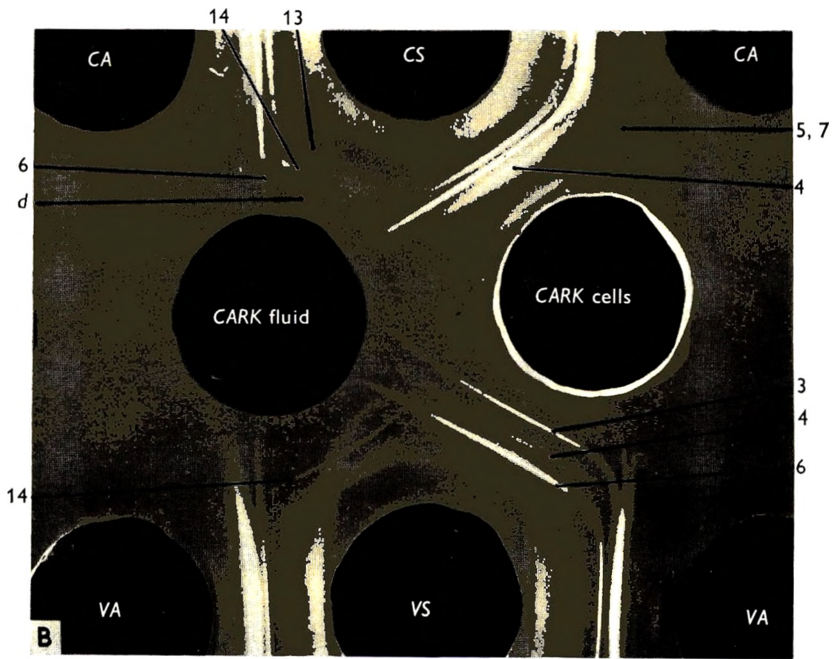
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EXPLANATION OF PLATES

Plate 1

A. Labelling of vaccinia soluble antigen produced on rabbit skin (*VA*) using antivaccinia serum (*VS*), egg-grown cowpox antigen (*CAE*) and heated preparation of vaccinia soluble antigen (*S*).

B. Labelling of cowpox soluble antigen produced in RK 13 cells (*CA*) using *VA*, *VS*, anticowpox serum (*CS*), and egg-grown white cowpox antigen (*WCAE*).

Plate 2

A. Vaccinia soluble antigens produced in RK 13 cells, labelled with the aid of *VA* and *VS*. RK 13 antigens have been separated by centrifugation after dialysis into buffer soluble (*VARK bs*) and water soluble (*VARK ws*) fractions.

B. Recognition of lpc 13 and 14 in the supernatant fluids (*CARK* fluids) from cowpox-infected RK 13 cells (*CARK* cells).

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Age susceptibility and excretion of *Salmonella typhimurium* in calves

By R. A. ROBINSON
Ruakura Agricultural Research Centre, Private Bag, Hamilton, New Zealand
AND K. I. LOKEN*
Department of Veterinary Bacteriology and Public Health, College of Veterinary Medicine, University of Minnesota, St Paul, Minn., U.S.A.

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The production and sale of surplus dairy calves may involve transport, marketing, slaughter, or rearing on a new property. Economics dictate that these transfers take place at a young age, usually 4–6 days. The problem of cross-infection at this time, particularly with salmonella organisms, has been recognized for some time (Anderson, Galbraith & Taylor, 1961; Robinson, 1966). Surveys (Anon. 1964) have shown that the incidence of clinical salmonellosis in purchased calves is considerably higher than in home-bred calves. It requires only a few calves excreting salmonellas on the farm to result in a high isolation rate of organisms following slaughter. Factors which contribute to this rapid build-up of infection include time of transport and holding, number of animals per unit area, partial colostrum deprivation, starvation conducive to extensive sucking amongst calves, and passage of calves through contaminated environments. De Jong & Ekdahl (1965), as a result of observations on the response of calves to *S. typhimurium* have suggested that if animals were retained on the farm of birth until they were at least 14 days of age, the prevalence of salmonella infection amongst calves entering the abattoir might well be reduced. Others (Williams Smith, 1966; Walton, 1966) have made similar suggestions in support of a ban on the movement of calves less than 6 weeks old.

Robinson (1966) has observed that calves may be exposed to infection within the first few hours of birth, either from a contaminated environment or from the adult cow. Excretion of salmonellas in the milk does not commonly occur unless the cow is clinically affected with salmonellosis, but gross contamination of the udder and teat surfaces with salmonellas can be demonstrated both in asymptomatic excreting animals and in recently calved cattle held in a contaminated environment.

In view of the above suggestions, it was, therefore, considered useful to compare the response of two groups of calves to several dose rates of *S. typhimurium* given orally at approximately 2 days of age, and at 14–21 days of age. These will be referred to as 'younger' and 'older' groups respectively. In these calves, the concentration of *S. typhimurium* in the faeces, the duration of faecal excretion and the

* Fulbright Research Scholar.

sites of recovery of the organisms after slaughter were examined. Calves were given doses of organisms which were unlikely to result in clinical disease. Throughout these trials calves were fed on whole raw milk. This would not be an economic feeding regime, but it was thought that data should be obtained on the response of calves under optimum nutritional conditions. The use of milk substitutes, partial colostrum deprivation and temporary starvation, are all additional factors which warrant separate investigation in the response of young calves to salmonella infections.

METHODS AND MATERIALS

Preparation of salmonella suspensions

Lyophilized cultures of the calf-passaged strain of *S. typhimurium* (phage-type 1) used by de Jong and Ekdahl were reconstituted in 0·5 ml. tryptose phosphate broth* and inoculated on 8 ml. nutrient agar slopes. These were incubated at 37° C. for 18 hr. and the bacterial growth was then washed off with 2 ml. broth and appropriate dilutions made in quarter strength Ringer solution. Viability counts were made by spreading 0·1 ml. volumes of dilutions on MacConkey agar plates in duplicate. A fresh freeze-dried culture was used for each group of animals. The required dose was given in 40 ml. sterile milk by mouth midway between morning and afternoon feeds.

Isolation of salmonellas

Apart from enumeration of the number of salmonellas in faecal samples, all materials were cultured for 24–30 hr. at 37° C. in tetrathionate broth, containing brilliant green at a final concentration of 1/100,000. Subcultures were made on freshly poured brilliant green (BG) agar with 8 mg. sulphadiazine added per 100 ml. agar. These plates were incubated for at least 24 hr. before picking suspicious colonies to a tube each of triple sugar iron agar (TSI) and lysine broth. BG plates with scanty growth were not discarded as negative until after 48 hr. incubation. All suspicious TSI tubes were confirmed serologically. The results of Nottingham's (1967) studies on six enrichment and plating media combinations showed the above method was the most efficient, obtaining 82 % of the total salmonella isolations from naturally infected calf tissues.

Faecal samples

All calves were sampled daily until slaughter; faeces for quantitative estimation of salmonellas was obtained by using sterile wooden spatulas, but later qualitative examinations were made using rectal calcium alginate wool swabs only. Enumeration of the number of salmonellas/g. of faeces (wet weight) was carried out using a dilution counting technique (Merselis, Kaye, Connolly & Hook, 1964). This method was used in preference to multiple tube enrichments as used by McCall, Martin & Boring (1966) because of greater ability to cover a wide range of counts

* Both Difco and Baltimore Biological Laboratories media were used throughout these studies.

with minimal media and laboratory examinations. The sensitivity of the method used, however, does not allow estimations below 100 orgs./g. faeces. A 10% suspension of faeces was made in quarter strength Ringer solution in a container with sterile glass beads and shaken for a few minutes. Tenfold dilutions in the same diluent were then made and 0·1 ml. of appropriate dilutions spread on BG plates with glass spreaders. Duplicate plates were spread from each dilution and a count of typical salmonella colonies made after 24–36 hr. Biochemical and serological identification was performed on randomly selected colonies. Double strength tetrathionate broth was added to one dilution (10^{-2}) and any salmonellas present recorded during the period that quantitative direct plating of faeces was in progress. Where the level of excretion dropped to 100 salmonellas/g. of faeces, alginate swabs were placed directly in 10–12 ml. enrichment broth.

Post-mortem samples

Calves were destroyed by exsanguination and, after skinning, carcasses were flamed with a butane burner. Asceptic precautions were taken in the removal of specimens, but following the recommendation of Kampelmacher, Guinée & Jansen (1964) all tissues were placed in water at 100° C. for about 6 sec. to reduce superficial contamination. The following tissues were examined: mandibular, parotid, suprapharyngeal, all mesenteric, caecal, colic, prescapular and precrural lymph nodes, spleen, liver (including gall bladder and hepatic lymph node) and kidney and tonsillar tissue. In addition, contents of rumen, abomasum, small intestine, caecum and rectum were examined for salmonellas. Spleen, liver, kidney and mesenteric lymph nodes were homogenized in a domestic blender and approximately 10 g. added to 100 ml. broth. Approximately 5 g. of contents of the gastro-intestinal tract were added to 100 ml. broth. In the early stages of this experiment, the small intestine was removed and divided into three or four sections and each portion homogenized. Later, in the calves dosed with 10^5 and 10^4 organisms, the less cumbersome method used by Williams Smith (1955) was adopted, viz. small sections were taken of duodenum, jejunum, and ileum about 3 in. long, the exterior flamed, slit longitudinally and a swab of the contents taken. The piece of intestine was washed in running water followed by soaking in 70% ethyl alcohol for 1 min. and again washed in water. The intestine was cut up finely with scissors and cultured in enrichment broth.

Experimental animals

Jersey calves were obtained from the dairy farms of Ruakura Agricultural Research Centre. To determine that these herds were free from salmonella infection at the time, swabs (Moore, 1948) were placed in the main drain leading from each dairy and cultured at weekly intervals. This procedure had been used previously (Robinson, 1966) to detect the presence of salmonella-excreting cows. Rectal swabs were also taken from all calves before experimental infection. All calves were left with the adult cow for 2 days after birth. Calves to be dosed at 14–21 days were weaned at 4 days of age and then reared in strict isolation. Calves were kept out of doors singly in pens of approximately 80–100 ft.^{2} and given whole

raw milk twice daily plus hay *ad lib*. Direct contact between calves did not occur. The only potential avenue of cross-infection was via the person sampling or feeding. Milk was checked occasionally for the presence of salmonellas. All feeding equipment (rubber teats, tubes and buckets) was rinsed in cold water followed by hot water after each feed.

Thirty-eight calves were given doses of viable *S. typhimurium* as set out in Table 1. The mean age of the older calves was 18 days when dosed.

Table 1. *Schedule of doses for older and younger calves*

Approximate dose	Number of calves	
	Younger	Older
10^6	12	11
10^5	3	4
10^4	4	4

RESULTS

There appears to be little or no information available about when viable organisms could be recovered from tissues following apparent cessation of faecal excretion. Whenever possible, at least 3 weeks of negative salmonella excretion was recorded before an animal was slaughtered, but eleven calves were killed after a shorter period, and three of the younger animals after less than a week of negative excretion. Occasionally a positive sample was obtained during a long run of negatives. Since calves were kept continuously in individual pens, these isolated positives may be the result of recent ingestion of a few organisms from the environment, or of the original infection in the calf; since it is not possible to be certain on this point no account was taken of these few positives in recording time to the last positive swab.

Table 2 shows, for individual calves, the time in days from dosing to slaughter, the time in days from dosing to the last positive rectal swab recorded, and the site of recovery of organisms after slaughter.

Duration of excretion

Overall analysis, using log (days + 1) to stabilize the variance, showed ($P < 0{\cdot}01$) that the younger calves excreted salmonellas for longer than the old animals, but indicated ($P < 0{\cdot}10$) that the ratio depended on the dose, being three and eight times longer for the 10^6 and 10^5 orgs/dose groups respectively. Little weight can be given to the corresponding ratio from the 10^4 orgs./dose group, as only one of the older calves recorded a positive, and that on the first day after dosing.

Sites of recovery of salmonellas

S. typhimurium was recovered at slaughter from 10/19 of the younger calves and 4/19 of the older animals. Among the younger calves receiving 10^6 organisms, salmonellas were found in four of the five killed after less than 3 weeks negative sampling compared with three of the remaining seven. The organisms were

Table 2. *Duration of faecal excretion, and post-slaughter recovery sites, of* Salmonella typhimurium

No. of orgs. in dose	Calf	Days from dosing to: Slaughter	Days from dosing to: Last +ve swab	Recovery sites of organisms after slaughter
		Calves dosed before 3 days of age		
10^6	0064	38	25	+ (mes. L.N.)
	0057	46	46	+ (mes. L.N., ileum, rectal contents)
	0005	56	20	+ (mes. L.N., caecal contents)
	0003	63	41	–
	0024	65	44	–
	0052	81	21	+ (phary. L.N., caecal contents)
	0002	103	72	–
	0058	130	125	+ (mes. L.N.)
	0051	139	115	–
	0067	151	135	+ (mes. L.N.)
	0001	230	168	–
	0004	236	194	+ (mes. L.N.)
Geometric mean			63·4	
10^5	0091	21	0	–
	0090	34	28	–
	0196	105	77	–
Geometric mean			12·1	
10^4	0198	82	8	–
	0193	82	52	+ (mes. L.N., caecal L.N.)
	0194	105	30	+ (mes. L.N.)
	0197	112	36	+ (mes. L.N., jejunal wall)
Geometric mean			26·2	
		Calves dosed at 14–21 days of age		
10^6	0070	34	12	+ (mes. L.N.)
	0026	37	27	–
	0031	44	12	–
	0032	44	1	+ (liver, duodenum)
	0080	44	15	+ (caecal L.N.)
	0025	50	25	–
	0016	64	16	–
	0023	64	26	–
	0066	64	32	–
	0022	69	51	–
	0065	140	129	+ (mes. L.N.)
Geometric mean			20·3	
10^5	0014	21	0	–
	0100	26	0	–
	0015	30	1	–
	0099	42	15	–
Geometric mean			1·4	
10^4	0067	19	0	–
	0069	19	0	–
	0056	26	0	–
	0007	27	1	–
Geometric mean			0·2	

recovered from the mesenteric, caecal and colic lymph nodes in twelve out of fourteen of the positive calves, indicating that in these studies the above sites appeared to be the most useful indicators of infection. This differs from the results of Guineé, Kampelmacher, van Keulen & Hofstra (1964) who considered that the mesenteric lymph nodes were less reliable indicators than the gall bladder.

Table 3. *Degree of faecal excretion of* Salmonella typhimurium *in calves dosed with approximately* 10^6 *living organisms*

Days after dosing	Excretion of *S. typhimurium*: Negative	Excretion of *S. typhimurium*: log orgs./g. < 2	< 3·5	< 5	≥ 5	Estimated median concentration (orgs./g.)
		Six calves dosed before 3 days of age				
1	—	1	1	4	—	$2{\cdot}7 \times 10^3$
2	—	—	1	4	1	$1{\cdot}5 \times 10^4$
3	—	—	2	3	1	$1{\cdot}3 \times 10^4$
4	—	1	1	2	2	$6{\cdot}1 \times 10^3$
5	1	—	2	1	2	$1{\cdot}8 \times 10^4$
6	1	—	2	1	2	$2{\cdot}5 \times 10^3$
7	1	1	—	4	—	$5{\cdot}3 \times 10^3$
8	1	2	2	1	—	$2{\cdot}7 \times 10^2$
9	—	2	3	1	—	$1{\cdot}3 \times 10^2$
10	1	3	1	1	—	$4{\cdot}6 \times 10$
11	1	3	1	1	—	$0{\cdot}3 \times 10$
12	2	2	2	—	—	$1{\cdot}2 \times 10^2$
13	2	2	2	—	—	$0{\cdot}9 \times 10$
14	3	2	1	—	—	—
		Six calves dosed 14–21 days of age				
1	—	2	3	1	—	$6{\cdot}7 \times 10^2$
2	—	2	3	1	—	$1{\cdot}1 \times 10^3$
3	1	3	2	—	—	$3{\cdot}0 \times 10^2$
4	1	3	2	—	—	$6{\cdot}0 \times 10^2$
5	1	4	—	1	—	—
6	1	2	2	1	—	$1{\cdot}2 \times 10^2$
7	1	3	2	—	—	$2{\cdot}0 \times 10^3$
8	1	1	4	—	—	$2{\cdot}1 \times 10^2$
9	3	3	—	—	—	—
10	3	3	—	—	—	—
11	5	1	—	—	—	—
12	4	2	—	—	—	—
13	5	1	—	—	—	—
14	4	2	—	—	—	—

However, when these workers examined all the mesenteric lymph nodes instead of only the caudal portions, their isolation of salmonellas doubled, indicating that the organisms appeared to be distributed uniformly over the whole mesenteric nodal system. Although the gall bladder and associated section of liver and hepatic lymph node were examined in our studies, *S. typhimurium* was recovered from these sites only once. In one calf (0197) salmonellas were recovered from the wall of the jejunum but not from the jejunal contents at that point. While it is possible

that the calves still harbouring salmonellas at slaughter could have recommenced excreting them, it is considered that the presence of organisms in the mesenteric lymph nodes only, represents the terminal phase of recovery from the original infection.

Salmonella concentration in the faeces

One measure of the infectivity of a calf to others is the number of salmonellas it sheds in its faeces. Therefore an investigation of the number of organisms excreted per gram of faeces was carried out. Table 3 summarizes the data from six younger and six older calves dosed with 10^6 *S. typhimurium*, for the first 14 days after dosing. The geometric means shown are estimates of the median level of excretion, and were obtained, using the tables of Sarhan & Greenberg (1956), by regarding samples rated at less than 10^2 orgs./g. as censored data from the same population as the estimable concentrations. On 4 of the first 7 days after dosing there were significantly ($P < 0{\cdot}05$, single tail) more younger calves with salmonella concentrations in the faeces greater than $3{\cdot}2 \times 10^3$ orgs./g. (i.e. antilog 3·5), and their median level was higher than for older calves on all days for which both estimates were available.

Counts were also made on faeces from calves dosed at the lower dose rates but only once was a count of greater than 10^2 orgs./g. detected. Calf no. 0196 dosed with 10^5 *S. typhimurium* at 2 days of age, continued to excrete for 14 days at 10^3 salmonellas/g. faeces.

DISCUSSION

Newell (1967) has stated that *Salmonella* infections are related to four independent factors, personal susceptibility, the risk of direct or indirect contact with a serotype, the number of organisms ingested, and serotype characteristics. In these studies we have endeavoured to examine one component of the first variable only, i.e. age. It was observed (Robinson, 1966) in an earlier trial that two calves dosed with 10^6 *S. typhimurium* at 2 days of age were more infective among their peers at 4 days of age than a similarly dosed pair were at 21 days of age. In these trials eight undosed in-contact calves in each group were killed after 24 hr. close confinement and transport with the dosed animals, and salmonella organisms were isolated from seven of the young group but from none of the older calves ($P < 0{\cdot}01$). This difference could be the result of either lower excretion rate of the dosed calves after 19 days infection or lowered susceptibility of the older calves. It would appear from the present studies that both factors operate. Calves dosed at 2 days of age are likely to be excreting few organisms at 21 days of age. If we accept that the number of organisms excreted following dosing is one indicator of susceptibility to infection, then the older dosed animals appear to be more resistant. Throughout these trials it was assumed that salmonella-excreting and salmonella-free animals would have come from separate farms before congregation. If, for instance, a 21-day-old calf was exposed to salmonellas 2 or 3 days before sale it is likely that it could contribute to cross-infection.

Other workers have demonstrated similar patterns of age resistance to enteric infections. Mushin & Dubos (1965) using an enteropathogenic strain of *Escherichia*

coli in mice found that the period of greatest susceptibility to oral infection extended from the day of birth until 2 weeks of age. After this time, colonization of the gastro-intestinal tract became more difficult, even when very large doses were administered on 3 consecutive days. They suggested that development in the gastro-intestinal tract of a microbiota which is antagonistic to *E. coli* may be responsible for this acquisition of resistance with age. Szanton (1957) has shown that new born infants infected with *S. oranienburg* during an outbreak continued to excrete the organism much longer than adults infected during the same outbreak.

A rapid increase in resistance to experimental salmonellosis with age has been demonstrated in both turkeys and chickens. Bierer (1960) has shown that a 90% mortality can be expected in turkey poults given varying doses of *S. typhimurium* within the first 6 hr. after hatching. The same doses (up to 10^9 viable organisms) given to birds at 72 hr. of age produced no effects. Severens, Roberts & Card (1944) and Williams Smith (1955) have also shown that a rapid increase in resistance to oral infection with *S. pullorum* developed in chickens during the first few days of life.

Diarrhoea is common among calves during the first 3 weeks of life, and if it is associated with salmonella excretion the risk of extensive environmental contamination is obvious. In these studies most calves exhibited diarrhoea at some stage, often accompanied by reduced appetite. The diarrhoea did not appear to be related to either dosing or high faecal salmonella counts. Apart from a reduction in milk intake no therapeutic measures were considered necessary.

It is recognized that calves from which salmonellas were recovered at slaughter may have recommenced faecal excretion following the pre-slaughter negative period, but it is difficult to eliminate the possibility of continuous re-ingestion of organisms from the environment. The persistence of these pathogens in calf pens and sheds is lengthy. Adinarayahan, Smyser & Roekel (1966) have observed that poultry excreting *S. heidelberg* cleared themselves of infection within a few days of being transferred to wire batteries; whereas their pen mates maintained in the original contaminated colony house continued to excrete this serotype intermittently. In the absence of re-infection therefore it would appear that *S. typhimurium* infection in calves is self-limiting and that most calves do not remain permanent shedders of the organism.

Williams Smith (1955) claims that there was no evidence that salmonellas multiplied within the lumen of the intestine. The recovery of *S. typhimurium* from the intestinal wall but not from the contents in one calf suggests that this is likely. Recent fluorescent antibody studies by Kent, Formal & Labrec (1966) of *S. typhimurium* infection in Rhesus monkeys show the organisms present in the surface epithelial cells, lamina propria and submucosa of the small intestine as well as in the associated lymph node.

SUMMARY

Two groups of calves were dosed orally with 10^4, 10^5, and 10^6 *S. typhimurium* at approximately 2 days of age and at 14–21 days of age. No obvious clinical signs were observed with this strain, but younger calves excreted the salmonellas in the

faeces for longer periods than older animals. The younger animals also excreted more salmonellas per gram of faeces in the first week following dosing. These observations may explain why salmonella cross-infection is likely to occur where very young calves are congregated. Following cessation of excretion of salmonellas in the faeces and subsequent slaughter of calves, examination of all the mesenteric, caecal, and colic lymph nodes showed these to be the most useful sites for recovery of salmonellas.

Provided good nutrition and hygienic conditions prevail, calves retained on their farm of origin for longer periods are more likely to recover from neonatal salmonella infections.

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A comparison of methods of measuring the persistence of neutralizing and haemagglutinin-inhibiting antibodies to louping ill virus in experimentally infected sheep

BY K. J. O'REILLY
Wellcome Research Laboratories, Beckenham, Kent
C. E. GORDON SMITH, D. A. McMAHON AND E. T. W. BOWEN
Microbiological Research Establishment, Salisbury, Wilts.
AND G. WHITE
Wellcome Veterinary Research Station, Frant

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In their preliminary study in 1960–1 on Camlarg Farm, Smith *et al.* (1964) reported that 25% of hoggs (yearling sheep) had lost haemagglutinin-inhibiting (HI) antibody less than 12 months after natural infection with louping ill, yet none had lost neutralizing antibody. The same authors (unpublished) found that among the hoggs exposed to louping ill infection between spring and summer of the years 1961–4, 20% of sera collected in the autumn had both neutralizing and HI antibody and a further 14% neutralizing antibody only. Williams & Thorburn (1961) showed that all of ten sheep had developed HI antibody by 8 weeks after experimental infection with louping ill virus. Thus the 14% of hoggs with only neutralizing antibody in the autumn had probably developed both types of antibody following infection but had lost HI antibody during the summer.

In surveys of sheep populations for antibody to louping ill, it is commonplace to find a higher proportion with neutralizing than HI antibody, presumably because the former is more persistent. For example, Smith *et al.* (1964) surveyed two flocks comprising 132 ewes and found that in March 68% had neutralizing but only 33% HI antibody; in June the percentages were 86 and 46 respectively. Findings like these present considerable difficulties in epidemiological interpretation.

In order to get a clear understanding of the fate of the neutralizing and HI antibodies, an experiment was designed, first, to determine the duration of the neutralizing and HI antibodies after experimental infection with louping ill virus and, second, to establish the effect of re-infection on the antibody pattern. Because this study could not be undertaken in an area where louping ill is enzootic as natural infections would confuse the results, it was carried out at the Wellcome Veterinary Research Station at Frant, Kent.

MATERIALS AND METHODS

Viruses

The strain of louping ill virus used originated from the Moredun Institute, Edinburgh. The sheep were infected with virus which had been maintained by serial intracerebral inoculation of sheep, and the virus used in the neutralization and HI tests had been passaged 35–40 times in the brains of adult mice.

Sera

Sera were inactivated at 56° C. for 30 min. and then stored at −20° C. Recent work (Verani & Gresikova, 1966; Gresikova & Sekeyova, 1967) has shown that heating of serum before kaolin extraction for non-specific haemagglutinin-inhibitors may generate an inhibitor not removed by kaolin. However, it does not appear to have caused any noticeable problem in this study.

Neutralization tests

These were carried out by two methods in mice 3 to 4 weeks old. In the constant virus/variable serum method (NAbD) dilutions of sera were mixed with an equal volume of about 100 mouse ICLD50 of virus diluted in fresh guinea-pig serum. The mixtures were incubated for 90 min. at room temperature and then inoculated intracerebrally (IC) into groups of six mice. The virus was titrated at $\sqrt{10}$-fold dilutions in 50% normal sheep serum. The results were computed using survival times by the method of Smith & Westgarth (1957). Serum dilutions showing a significant difference from the controls at the 5% level were recorded as positive. In the constant serum/variable virus method (NAgD) the tests were similar except that undiluted sera were mixed with equal volumes of four tenfold dilutions of virus in fresh guinea-pig serum. The log neutralizing index was the difference between the log ICLD50 of the virus after incubation with test sera and the log ICLD50 of the virus after incubation with antibody-free serum. The end-points were calculated by the method of Reed & Muench (1938).

Haemagglutinin-inhibition (HI) tests

Haemagglutinating antigens were prepared by the sucrose–acetone method (Clarke & Casals, 1958) from the brains of moribund baby mice, infected with the Moredun strain of virus. Using the microtitre equipment (Cooke Eng. Co., Alexandria, Va.) and the method of Sever (1962), the test was carried out in two ways, comparable to the neutralization tests. The sera were extracted with kaolin (Clarke & Casals, 1958), heated at 56° C. for 30 min., and then absorbed with goose erythrocytes before testing since high titres of goose agglutinins are quite common in sheep sera (Smith, 1967). After the serum–antigen mixtures were incubated at room temperature for 60 min., 0·25% goose erythrocytes were added in phosphate buffer, which adjusted the reaction to pH 6·3. Haemagglutination was allowed to occur at room temperature (Clarke & Casals, 1958). In the constant antigen/variable serum method (HAbD) twofold dilutions of extracted serum were tested against

8–16 units of haemagglutinin as described by Smith *et al.* (1964). In the constant serum/variable antigen method (HAgD) 1/10 extracted serum was tested against a twofold dilution series of antigen concentrations from 64 to 1 unit.

Statistical analysis of antibody titres

This was carried out by Mr S. Peto and Mr B. Maidment of the Microbiological Research Establishment, to whom we are greatly indebted. Correlation coefficients between HAbD and HAgD, and between NAbD and NAgD, were calculated and their significance levels determined. Regression coefficients of log titre on both time and log time were obtained by computer; in general a better fit on log time was found for HAbD and HAgD while no clear-cut preference could be demonstrated for NAgD and NAbD. Log time was therefore adopted as it provided the best opportunity for a consistent comparison of the eight curves. However, the die-away with NAbD and HAbD following infection was markedly steeper in the earlier than the later stages and a hyperbolic curve (log titre *v.* reciprocal time linear) would have fitted these two much better. The weighted means of the regression coefficients were calculated and adapted for heterogeneity. Combination of these weighted means was justified only for HAbD and HAgD. The variability in response between animals was studied by comparing slopes and by plotting $\log_2$ titres *v.* time and superimposing results following infection on results following re-infection.

Animals

In May 1962, five Dorset Horn hoggs received a dose of louping ill vaccine* after blood sampling. They were bled 21 days later when they were infected subcutaneously (SC) with 10^5 mouse ICLD50 of louping ill sheep brain virus. Subsequently they were bled at intervals of approximately 4 weeks. Two months after the vaccination of the five hoggs, a further nine hoggs were treated similarly. Three sheep died from causes other than louping ill in April 1963.

In February 1964, all the surviving animals were infected SC with the same dose and strain of virus that they had received 19–21 months previously. One died of a metabolic disease in December 1964.

A number of these sheep lambed once, twice or three times during the course of the experiment and an opportunity was thus afforded to study the fall of maternal antibody in the lambs.

RESULTS

No sheep had either neutralizing or HI antibody before or 21 days after vaccination. Detailed analysis is limited to 11 animals who survived the entire experiment and whose sera have been fully tested.

Neutralizing antibody

Two recognized methods of testing for neutralizing antibody were used. As many as possible of the sera from a single sheep were tested at the same time.

* Burroughs Wellcome and Co., London.

Constant serum/variable virus method (NAgD)

The geometric mean titres and ranges in sera from the 11 sheep are shown in Fig. 1. Regression analysis showed that the weighted means of regression co-efficients adapted for heterogeneity were $+0{\cdot}25 \pm 0{\cdot}065$ following infection and $-0{\cdot}11 \pm 0{\cdot}054$ following re-infection. Thus there was no fall in NAgD antibody during the 81 weeks following infection (in fact a small gain) and a very little, if any, fall following re-infection. No real change was detected in NAgD antibody from about 4 weeks after infection until the end of the experiment.

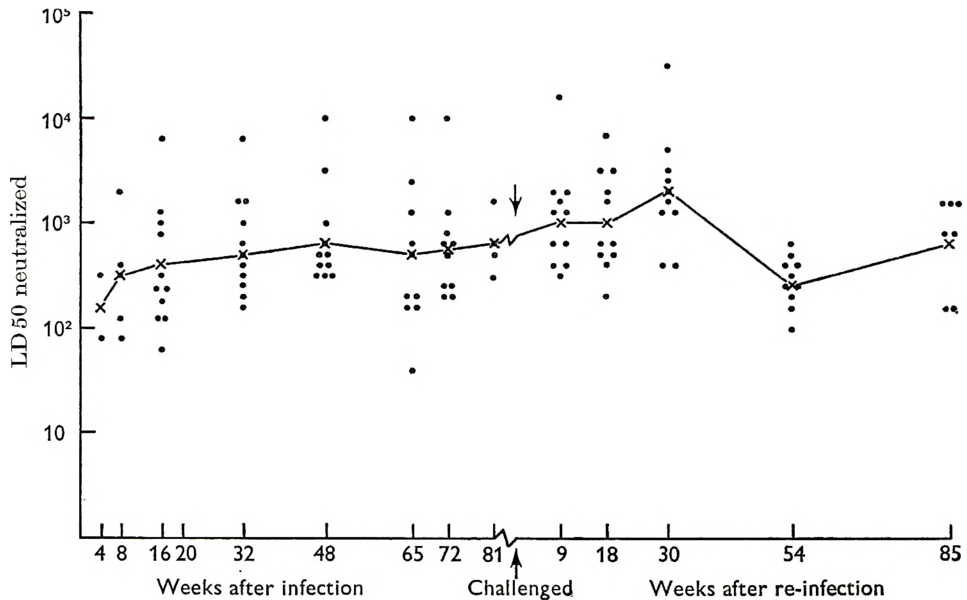

Fig. 1. NAgD. The geometric means and ranges of log neutralizing indices of undiluted sera against louping ill virus.

Constant virus/variable serum method (NAbD)

Most of the same sera were retested by this method (Fig. 2). The corresponding mean regression co-efficients were $-0{\cdot}39 \pm 0{\cdot}069$ following infection and $-0{\cdot}81 \pm 0{\cdot}10$ following re-infection. The geometric mean titres show that there was a rapid initial fall of rather more than fourfold in the first 20 weeks after infection and relatively little change thereafter. Following re-infection the geometric mean titre rose to the level following infection but fell to an ultimately lower level.

The highest titres of NAbD antibody following infection were 1/80 in four animals, 1/20 in two and 1/5 in three. One animal failed to develop significant NAbD antibody, although it had consistent NAgD titres of $> 2{\cdot}0$ log throughout and another similar animal had a barely detectable NAbD titre only on the 68th week after infection. By 64–68 weeks after infection, five of the nine animals with initial NAbD antibody had shown no fall in titre; three had fallen fourfold and and one 16-fold. By 73–81 weeks, seven of the nine had fallen fourfold from the highest level and one 16-fold; only one of the nine no longer had detectable NAbD

antibody. Nine weeks after re-infection six of the nine animals had reverted to their highest titre following first infection, two had higher titres and one lower. The animal with only a barely positive titre following infection showed a rise to 1/20 following re-infection. The remaining animal was not re-infected and is not considered further. Fifty-four weeks after re-infection, two animals had no loss of titre, five had a fourfold loss and two a 16-fold loss. By the 85th week antibody was no longer detectable in two of the six animals tested.

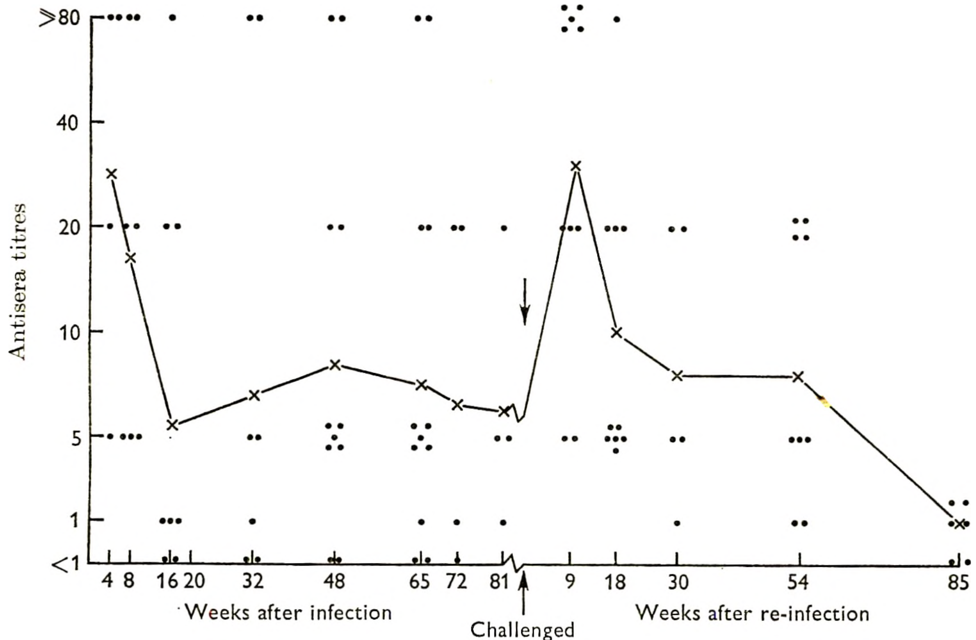

Fig. 2. NAbD. The geometric means and ranges of maximum serum dilutions neutralizing 100 mouse ICLD50 of louping ill virus.

Haemagglutinin-inhibiting antibody

Constant virus/variable serum method (HAbD)

Three to eight weeks after infection all except one sheep had significant (> 1/10) HAbD antibody (Fig. 3). This sheep developed no HAbD until after re-infection although it had all three other antibody types. The weighted mean regression coefficient following infection was $-1{\cdot}25 \pm 0{\cdot}19$. The geometric mean titres show a rapid initial fall of about $3\frac{1}{2}$-fold during the first 20 weeks and a much slower fall thereafter. At the 32nd week only five sheep had significant antibody titres which were on average fivefold lower than the corresponding titres of these sheep at the 8th week. By the 72nd week after infection only one sheep had detectable HAbD antibody, but 9–14 weeks after re-infection all but one sheep had HAbD antibody. Of nine sheep in which titres 8 weeks after infection could be compared with titres 9 weeks after re-infection, four had similar titres on both occasions, five had titres after re-infection lower by at least fourfold, and the animal with no HAbD antibody following infection developed a titre of 1/20. By 30 weeks after

re-infection, HAbD antibody had become undetectable in two of the animals and, between 54 and 86 weeks, three more animals became negative. At 86 weeks, four animals had titres which had been unchanged from the 9th week after re-infection. The regression coefficient following re-infection was $-0{\cdot}40 \pm 0{\cdot}10$, indicating a much slower rate than following infection.

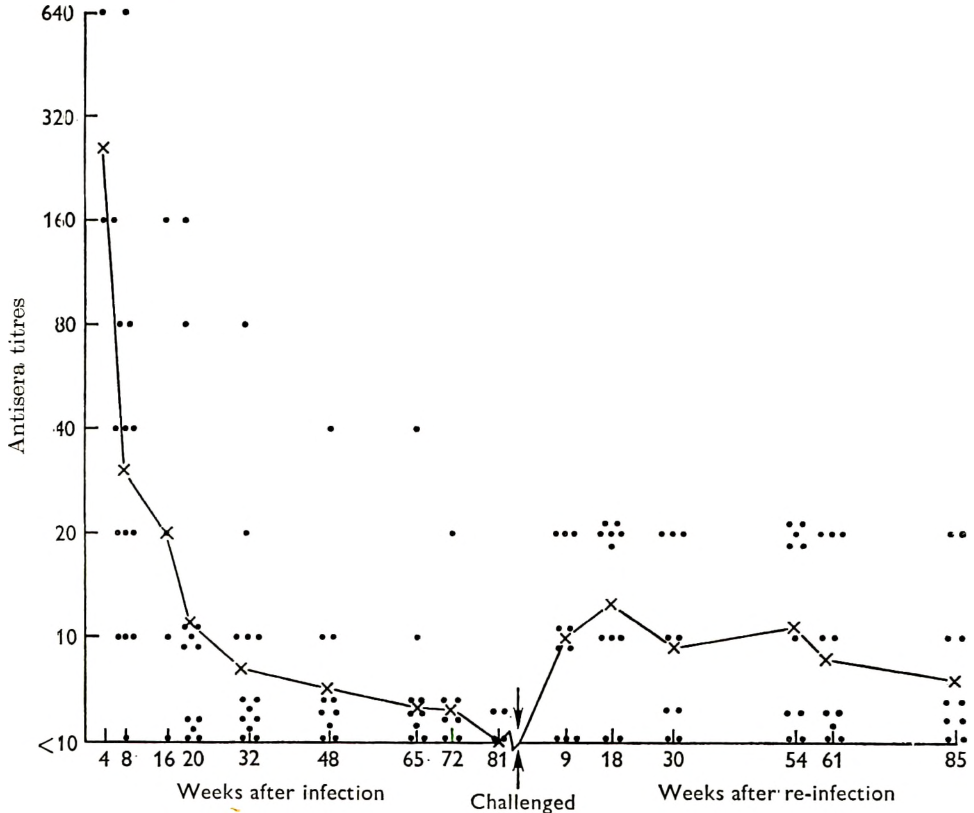

Fig. 3. HAbD. The geometric means and ranges of maximum serum dilutions inhibiting 8–16 units of haemagglutinin.

Constant serum/variable virus method (HAgD)

All sera from animals 3 weeks after infection inhibited at least 64 haemagglutinin (HA) units, and all at 8 weeks at least 16 HA units (Fig. 4). The regression coefficient following infection was $-1{\cdot}06 \pm 0{\cdot}12$. The geometric mean titres show a roughly linear fall of about eightfold during the 81 weeks. At the 72nd week after infection, sera from only two animals failed to inhibit at least 4 HA units. Nine weeks after re-infection, all the sera except two inhibited more than 16 HA units and none less than 8 HA units. Compared with titres 8 weeks after initial infection, the titres 9 weeks after re-infection differed by more than twofold in only two animals. By 86 weeks after re-infection all animals still had significant HAgD antibody; sera of four inhibited 4–8 units and sera of five inhibited 16–32 HA units. The regression co-efficient following re-infection was $-0{\cdot}61 \pm 0{\cdot}10$, indicating a slower rate of fall than following infection.

Relationship between the tests

The correlation coefficient between the results of the two HI tests were highly significant (0·79 following infection with 75 degrees of freedom (D.F.); 0·70 following re-infection with 72 D.F.) and combination of their corresponding weighted means

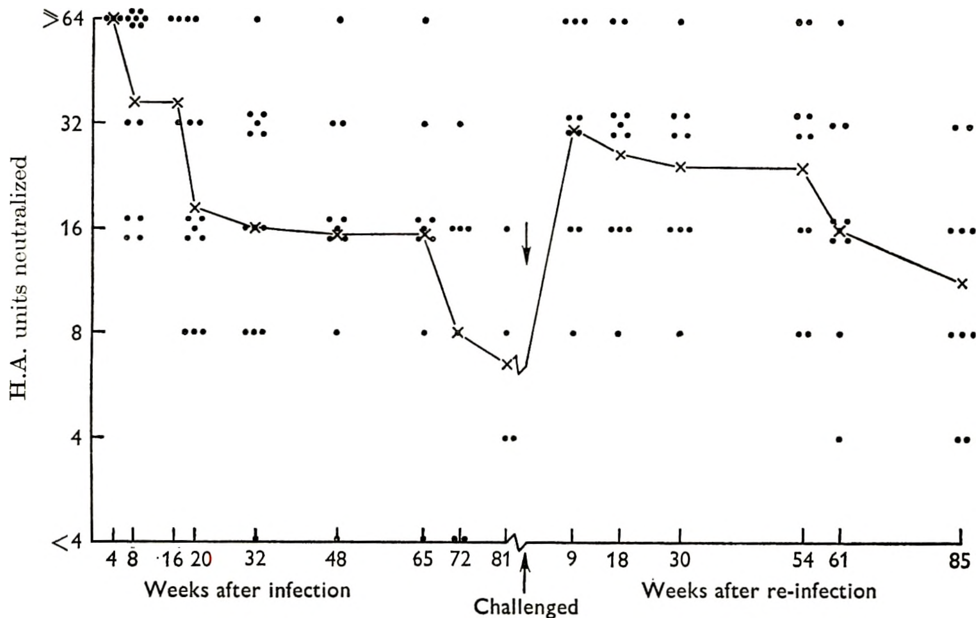

Fig. 4. HAgD. The geometric means and ranges of the maximum number of HA units neutralized by 1/10 dilution of sera.

Table 1. *Variability to responses illustrated by the slopes of the regressions of titre on log time*

Antibody	Following infection or re-infection	No. of slopes: Positive	Zero	Negative
HAbD	Inf.	0	1	10
	Re-inf.	3	2	6
HAgD	Inf.	1	1	9
	Re-inf.	0	2	9
NAbD	Inf.	1	2	8
	Re-inf.	0	1	10
NAgD	Inf.	7	0	4
	Re-inf.	3	0	8

of regression coefficients showed $-1·17 \pm 0·11$ following infection and $-0·48 \pm 0·07$ following re-infection. Thus, by both tests the rate of fall in HI antibody following first infection was significantly faster than following re-infection. No significance could be attached to the correlation coefficients between the results of the neutralization tests.

There was considerable variation between individual sheep as shown (Table 1)

by the distribution of positive and negative slopes of these ranges. A high proportion of positive slopes was seen only with NAgD. Four sheep had notably higher HI titres following infection than re-infection at corresponding times but the reverse was true for two other sheep.

Maternal antibody

Lambs born to the ewes in this experiment were bled after birth and at intervals where possible. The mothers were also bled on or near the date of the birth of the lamb.

The HAbD and NAbD antibody titres of sera taken from 12 lambs within 6 days of birth were greater than the corresponding maternal antibody titres in 11 with HAbD and six with NAbD (Table 2).

Table 2. *Relationship of lamb antibody titres at 1 week after birth and ewe antibody titres at or near parturition*

Ewes			Lambs			
	Antibody titres			Antibody titres		
Number	HAbD	NAbD	Date of birth	HAgD	HAbD	NAbD
31	< 10	5	Nov. 1964	8	10	*
83	< 10	5	Nov. 1964	† 16	20	20
				16	20	20
85	< 10	5	Sept. 1963	† 64	20	5
				64	20	20
33	< 10	20	Nov. 1964	† 64	20	20
				32	20	80
34	10	5	Oct. 1964	32	20	> 20
32	20	20	Nov. 1964	† 64	20	20
				> 64	40	> 20
85	20	20	Nov. 1964	32	80	5
90	40	20	May 1964	NT	80	20

* Antibody present in undiluted serum.
† Twin lambs. NT = Not tested.

The decay pattern of the antibodies in the ewe and lamb sera appeared to be similar; NAbD antibody in lambs persisted longer than HAbD antibody in 14 of 21 animals examined. However, NAbD antibody persisted longer than HAgD antibody in only five of 13 animals. HAgD antibody lasted longer than HAbD antibody in seven of the 13 lambs but neither type of HI antibody was found after the disappearance of NAgD antibody.

DISCUSSION

In sheep vaccinated, then infected, with louping ill virus, neutralizing antibody is produced and, if measured by the constant serum/variable virus (NAgD) method, persists without significant change of titre for at least 1·5 years. Re-infection at this stage causes no significant change in NAgD antibody levels, which persist for

at least another 1·5 years. If the measurement is by the constant virus/variable serum (NAbD) method the antibody falls in titre after reaching its peak, so that on the average the titre is fourfold less at the 16th week and thereafter remains constant until at least 1·5 years after infection. One animal failed, and one almost failed, to develop detectable NAbD antibody, although the three other tests demonstrated antibody. After re-infection the NAbD antibody levels rose to those following infection and the animal which had almost failed to produce antibody after infection responded after re-infection. NAbD titres fell significantly more rapidly following re-infection than following infection but at least two-thirds of the animals still had significant antibody about 1·5 years after re-infection.

Although HAbD titres fell more rapidly than HAgD titres following infection no significant difference in the rate of fall in titre between the two HI tests could be established. Both types of titre fell significantly more rapidly than the neutralizing antibody titres following infection; and the HI titres fell more than twice as rapidly following infection than following re-infection. However, because of the greater sensitivity of the HAgD test, significant antibody persisted longer; 7 months after infection HAbD was detectable in only half the animals, while nearly all still had HAgD antibody; after 1·5 years only about one-third had detectable HAbD antibody but 90 % had HAgD antibody. After re-infection 70 % showed an antibody response to either test; 1·5 years later about half the animals had detectable HAbD antibody but all had detectable HAgD antibody.

These results can be compared with those of Williams & Thorburn (1961), who inoculated five sheep from a flock free from louping ill with approximately 10^6 mouse LD 50 of louping ill virus. None developed detectable HI antibody, yet ten other sheep which were inoculated with vaccine and virus produced antibody. This might suggest that the vaccine was necessary for the stimulation of this antibody. However, Smith *et al.* (1964) failed to find antibody following the use of vaccine and in their preliminary study at Camlarg Farm reported that unvaccinated but naturally infected hoggs did produce HI antibody. O'Reilly *et al.* (1965) found that in sheep inoculated with Langat virus the presence or absence of HAbD antibody to Langat virus did not influence the development or titre of louping ill HAbD antibody when these sheep were later exposed to natural louping ill infection.

Of the ten sheep which Williams & Thorburn vaccinated and infected, three lost their HI antibody within 4 months of the infection. During this same period these sheep were each inoculated with a total of six injections of louping ill virus. None of these sheep which lost their HI antibody regained it as did 70 % of those reported here. However, there were differences in the technique of the HI test used.

Previously in work with louping ill, the NAgD method has been generally used for measurement of neutralizing antibody and the HAbD method for measurement of HI antibody. In epidemiological studies this has, for now obvious reasons, led to the common finding of higher prevalences of neutralizing than HI antibody in enzootic areas.

A comparison of the loss of HAbD antibody demonstrated in hoggs between September 1960 and March 1961 following an epizootic in spring 1960 (Smith *et al.*

1964) with the experimental results reported here shows reasonable agreement ($P = 0{\cdot}20$). In the same study, the HI antibody found in March in 4- to 6-year-old sheep (before infection in that year) probably indicated repeated exposure to louping ill in the preceding years. For the detection of the maximum number of long-past infections there is no doubt that the NAgD type of neutralization test is best, but for the detection of recent infections the HAbD type of HI test is probably most suitable. For diagnosis by demonstration of the largest rise in antibody during the course of a louping ill infection in sheep, reasons of economy recommend an HI test and either could be used. However, the HAgD test appears to be more sensitive. If diagnosis is to be attempted by detection of a falling titre, either of the antibody dilution tests would suffice.

Most of the lambs 6 days after birth had higher titres of antibody than their mothers; 10 of 12 had at least twofold more HAbD and 6 of 12 at least fourfold more NAbD than the ewes. These findings are in agreement with those of Sterne *et al.* (1962), who stated that newborn lambs may have two to four times more *C. welchii* β- and ϵ-antitoxins than the ewes. This phenomenon was explained by Howie, Barr & Glenny (1953), who reported that there is almost a tenfold concentration of diphtheria antitoxin in the colostrum of ewes.

SUMMARY

Sheep, after infection with louping ill virus and after re-infection with the same strain of virus 19–21 months later, were bled at intervals and their sera examined for neutralizing and haemagglutinin-inhibiting antibodies. Each antibody type was measured by the constant serum/variable virus and constant virus/variable serum methods. The persistence of each type of antibody and its significance in epidemiological studies is discussed. The relationship of antibody levels in ewes and their lambs was also examined.

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Thermal comfort during surgical operations

BY D. P. WYON*, O. M. LIDWELL AND R. E. O. WILLIAMS
Central Public Health Laboratory, Colindale Avenue, London, N.W. 9 and Wright-Fleming Institute, St Mary's Hospital Medical School, Paddington, London, W. 2

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In spite of much recent discussion and complaints by surgeons that operating rooms are often hot and uncomfortable no direct studies appear to have been made of this environment in Great Britain. Bedford (1936) found that most people in very light industrial work preferred an indoor temperature of 65° F. in winter. A later investigation in a similar environment in summer (Hickish, 1955) indicated a slightly higher preferred temperature, 67·5° F. American surgeons (Houghten & Cook, 1939) appear to prefer a substantially higher temperature, 73° F. but this is also true of North American people generally. However, a recent study among office workers in Great Britain (Black & Milroy, 1966) gave an optimum temperature for comfort as high as 72° F.

Operating room temperatures have often been kept fairly high in the supposed interest of the patient. More recently it has been assumed that this is not necessary (Angus, 1959). Some current physiological studies, however (Mr H. F. Lunn, personal communication) suggest that heat loss from ordinary adult patients during surgical operations is significant. Full control of patients' heat loss by adjustment of the thermal environment in the operating room is unlikely to be practicable, and, if such control is required, it must and can be achieved by direct methods applied to the patient himself. The present study has been concerned solely with the comfort of the operating room staff and was devised to provide guidance for the design of operating suites in the British Isles.

Comfort is a subjective assessment related to, but not entirely determined by, the thermal effects of environment; a good general discussion is given by Bedford (1964). In particular, the perception of comfort, or more accurately of discomfort, is affected by activity, both physical and mental, by clothing, acclimatization and personal idiosyncrasies. These factors may lead to varying responses by the same individual on different occasions in the same physical environment as well as to different responses by different individuals. It is important also to realize the ephemeral element in assessments of comfort. As habits and tolerance change so will the acceptable range of environmental conditions. However, such changes are slow and the factors relevant to the perception of comfort remain relatively constant within a particular situation.

* Now at: Statens Institut för Byggnadsforskning, Valhallavägen 191, Stockholm 27, Sweden.
Requests for reprints should be sent to O. M. Lidwell at the Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

METHODS

Organization

A large number of comfort studies have been carried out by repeated observation on a small number of individuals in an experimentally varied environment. This is a satisfactory method for a fundamental study of the relative effects of different environmental variables. It was not considered suitable for this investigation, since we were concerned with comfort assessments under normal working conditions and with the full range of interpersonal variations to be found in practice.

We therefore visited thirty separate operating suites in twenty-eight different hospitals and made environmental observations and questioned the working staff in eighty-five operating rooms in use. In order to allow for the possible effect of season of the year and to study as wide a range of operations as possible, each suite was visited over a period of 3 days (an average of 5 half-day working sessions) in 1 week, on three separate occasions during a year at approximately 4-month intervals. The hospitals were chosen to provide as wide a range of environmental conditions as possible, and therefore included a proportion of older suites with unsatisfactory working conditions. Teaching hospitals and specialized units were avoided since we wished to study conditions in general surgery.

The visits were made by one of us (D.P.W.) accompanied by a nurse. Both changed into operating room clothing and were present in the operating room throughout the whole of each session that was studied. The minute-to-minute progress of a session was recorded, with full details of the number, type and length of operations performed, the number of staff present and the amount of movement in and through the theatre. This part of the study will be the subject of a separate publication. Any occurrence, such as a mishap or a surgical complication, that could have affected the subjective perception of the environment, was recorded. The details of clothing, sex, age, build, etc., for each person were obtained either by observation or by interview, and recorded. At frequent intervals the four principal environmental variables, air temperature, radiation temperature, air velocity and relative humidity were measured at the table and at the periphery of the room, using specially constructed equipment described elsewhere (Lidwell & Wyon, 1968). The apparatus was small and easily portable and its use did not involve any preparation of the operating room in advance.

Between operations, at intervals of about 2 hr., the surgeon, his assistant, the scrubbed-nurse and the anaesthetist, referred to as the operating-room table staff, were interviewed briefly in a standard way. The sequence of questions included separate groups on thermal comfort at each of three levels—head, body and feet. Bedford's seven-point scale of thermal sensation (Bedford, 1936) was used, but it was found convenient to assign a different set of numerical values to the 7 points on the scale, namely: 0, much too cool; 1, too cool; 2, comfortably cool; 3, comfortable; 4, comfortably warm; 5, too warm; 6, much too warm. An estimate was also made of the extent to which the subject was sweating on the forehead during the operation before questioning. Visible sweating was recorded on a 5 point scale:

0, forehead dry; 1, forehead damp; 2, forehead wet showing beads of sweat; 3, forehead wet and needing wiping; 4, clothing also visibly wet. Additional questions referred to 'stuffiness', air movement, noise, humidity, movement of staff and lighting. Replies were coded in a standard way, and care was taken to ensure that the subjects were unaware of the actual thermometer reading in order to avoid obtaining a stereotyped response related to the numerical value of the temperature.

This procedure provided information on all aspects of the session that might have influenced subjective thermal comfort, while taking up the minimum amount of the subject's time. No attempt was made to alter in any way the conditions that were found. Thus the readings are both representative and 'normal' in the sense that the subjects in each suite were used to them. Ample variation for the purpose of analysis was found among the suites visited, although conditions were more often too hot than too cold.

Data recording

In view of the very large number of units of information recorded, some method of mechanically assisted analysis was essential. It would have been preferable to have recorded all the information directly in coded form using a single card for each person-occasion. A great deal of subsequent labour, with the accompanying possibilities of transcription error, would then have been avoided. However, the situation in an operating room during a working session makes all note-taking difficult and this, coupled with the short time usually available for making the observations, made it necessary to use separate field sheets for the different categories of information, e.g. environmental particulars which were usually common to several subjects, subject responses, clothing and other personal details, etc. The basic assembly of information for analysis was that pertaining to a single person-occasion consisting of the results of the interview together with all the other recorded data relevant to this. Over 2500 such records were obtained in the course of 2 years' work between November 1963 and December 1965.

The data recorded on the individual field sheets were worked up so as to produce standardized summaries of the information relevant to each interview. This information together with the interview responses was then transferred on to a coding card, one card being used for each person-occasion. To minimize error in transcription no coding of the data recorded on the field sheets was done at this stage. Coding into integral numerical form suitable for the subsequent analysis was done later and the values written into a second set of spaces provided on the card. Accommodation of all the information finally required needed most of the 160 fields, corresponding to the number of columns on two standard-form punch cards, and the operators were able to punch each pair of cards directly from this second set of spaces.

Paired cards are satisfactory for computer input but present difficulties in carrying out mechanical cross-tabulation. In order to reduce these difficulties, some of the data were included on both of a given pair of cards. Subsequently a third card was prepared and punched by computer. This carried a selection of the data that previous analysis had shown to be of particular interest, together with

predicted responses computed from one or other of the regression equations obtained from this analysis.

Analysis

The main bulk of the analysis was carried out by computer, but before the material was submitted to this process mechanical sorting of the cards was used to determine certain preliminary characteristics of the data. The range and distribution of the values of the principal variables was obtained. The continuous and multi-valued variables, including the subjective responses, were found to be distributed sufficiently close to normality to justify analysis by standard methods without transformation of the variables. The non-metric variables were treated as described by Lidwell (1961). As the cooling effect of air movement on a cylindrical or near cylindrical object, such as the human body, is closely proportional to the square root of the air velocity, and as this function has been used in a number of comfort indices (e.g. Webb, 1959; Lee, 1958), we also used it in our analyses. The limited range of air velocity encountered in the operating rooms and the small effect produced by these variations on the comfort responses meant that, from our own observations, we were unable to justify the choice of any particular functional relation between air velocity and comfort response.

Two-way sorting was also used to explore the possibility of strong interactions between any pair of variables. This revealed that the extent to which the comfort vote varied with relative humidity was greater at higher than at lower ambient temperatures. The variation of the comfort vote with differences in the pressure of water vapour in the atmosphere, however, appeared to be independent of temperature over the range studied. All subsequent analysis was therefore carried out using water vapour pressure as the index of humidity.

The general analysis method employed was to determine a linear regression equation between the response as dependent variable and those other variables that gave significant regression coefficients. We included a general comfort response (T_0) obtained by summation of the numerically coded values of the responses for the head, trunk and feet (T_1, T_2 and T_3) and dividing the result by 3, i.e.

$$T_0 = (T_1 + T_2 + T_3)/3.$$

The programme employed was the BMD 02 R (Biomedical Series, 1965) sequential regression analysis by which variables are added to the regression analysis in the order in which they make the biggest reduction in the residual sum of squares until the effect of adding the next variable falls below a specific level of significance.

There were a large number of factors recorded that seemed unlikely to be of major significance or that were only recorded for a proportion of person-occasions. These were not included in the main analysis but were examined subsequently. A third card was prepared, by computer method, for each person-occasion on which were recorded the expected votes, calculated from the regression equations determined by the preceding analysis, together with the actual votes recorded and the data relating to these other factors.

Sequential regression analyses, using the same BMD 02 R programme, were carried out using the differences between the calculated and recorded votes as the

dependent variables. These analyses were carried out on the whole of the data, or on a part where the factor in question had only been recorded on a proportion of occasions.

RESULTS

The mean and standard deviation for the major variables recorded are given in Table 1. It can be estimated from these figures that temperatures exceeding 75° F. were recorded on about 25% of occasions and that nearly 20% of the recorded

Table 1. *Mean and standard deviation of the major variables*

Variable	Mean	Standard deviation
1. Ambient temperature (° F.): head	72·6	4·0
trunk	72·3	4·0
feet	71·8	3·9
2. Water vapour pressure (mm. Hg)	10·2	2·4
3. Radiation (° F. excess over ambient): head	4·3	2·8
trunk	2·1	1·5
feet	1·6	1·6
4. Air movement $(\text{ft./min})^{1/2}$: head	4·59 (≡ 21 ft./min.)	1·16
trunk	4·77 (≡ 23 ft./min.)	1·21
feet	5·51 (≡ 30 ft./min.)	1·50
5. Age (yrs.)	38·6	9·3
6. Sex (proportion female)	0·364	—
7. Race (proportion European)	0·836	—
8. Build	2·820	0·78
9. Surgeon (proportion)	0·228	—
10. Surgical assistant (proportion)	0·250	—
11. Scrubbed nurse (proportion)	0·281	—
12. Anaesthetist (proportion)	0·241	—
13. Spring (fraction responses Mar.–May)	0·260	—
14. Summer (fraction responses June–Aug.)	0·187	—
15. Autumn (fraction responses Sept.–Nov.)	0·292	—
16. Winter (fraction responses Dec.–Feb.)	0·261	—
17. Stay in room (hours up to time of questioning)	2·00	1·11
18. Stress	1·46	0·73
19. Proportion wearing glasses during operation	0·287	—
20. Comfort: for head (T_1)	3·69	1·14
for trunk (T_2)	3·51	1·17
for feet (T_3)	3·31	0·97
21. Comfort overall ($T_0 = (T_1+T_2+T_3)/3$)	3·50	0·94
22. Degree of visible sweating on forehead (P)	0·58	0·62

Variables 6, 7, 9–16 and 19 are non-metric so that a standard deviation is not an appropriate measure of dispersion.

Build was recorded in 5 steps from 1, fat, through 3, normal, to 5, thin. The operations were recorded in three categories: 0, minor; 1, normal; 2, major. The coded figure for stress was then taken as the time weighted average of this over the period spent in the operating room up to the time of questioning.

Comfort and sweating were scaled as described in the text.

votes indicated that the individuals concerned felt too hot or much too hot, e.g. the upper boundary for comfort is a vote of 4·50, treating the vote as a continuous variable. The mean value of the response at head height, T_1, was 3·69 with a

standard deviation of 1·14. A response of 'too hot' or 'much too hot' therefore exceeds the mean value by 4·50–3·69 = 0·81 which is equal to 0·81/1·14 = 0·71 times the standard deviation. For a normal distribution approximately 24% of instances depart further in one direction from the mean than this. The mean humidity recorded corresponds very closely to a relative humidity of 50% at the average temperature recorded.

Table 2. *Coefficients of the regression equations for prediction of the subject's response. Environmental variables only*

Variable	Response				
	T_1	T_2	T_3	T_0	P
1. Ambient temperature (° F.)	0·126	0·134	0·091	0·118	0·038
2. Water vapour pressure (mm. Hg.)	0·066	0·076	0·048	0·062	0·033
3. Radiation (° F.)	0·037	0·095	0·050	0·079	0·025
4. Air movement (ft./min.)$^{\frac{1}{2}}$	NS	−0·073	−0·030	−0·058	NS
Constant	−6·340	−6·848	−3·669	−5·636	−2·694
Multiple correlation coefficient	0·504	0·503	0·394	0·548	0·331
Correlation coefficient with ambient temperature only	0·466	0·452	0·366	0·500	0·262

The regression equations are of the form

$$\text{predicted response} = a + b_1x_1 + b_2x_2 + \ldots$$

and the table gives the values of the coefficients b_1, b_2, etc., and of the constant a. All the coefficients listed exceed twice their standard errors. NS indicates that the variable was not entered into the regression, its effect on the residual sum of squares being insignificant.

T_1, T_2, T_3 are the scale values of the interview response for comfort of head, trunk and feet respectively.

T_0, the general comfort response, $= (T_1 + T_2 + T_3)/3$.

P is the extent of visible sweating on the forehead according to the scale described in the text.

In Table 2 the coefficients of regression equations between the comfort responses and the principal environmental variables are listed. By comparison with the range of values encountered for these variables (see Table 1) and by inspection of the correlation coefficients listed, it is apparent that ambient temperature is by far the most important in determining the comfort response and that air movement in particular played a very small part. This is perhaps a consequence of the very small areas of skin exposed when wearing operating room dress.

The comfort response was significantly affected by a number of other recorded variables of either a personal or environmental nature. The coefficients obtained for the regression equations, on the same comfort responses as those included in Table 2, are given in Table 3 for all the major recorded variables. The increase in the precision of these equations considered as predictive instruments is only very slight compared with the equations defined by the coefficients given in Table 2. For example, the general comfort response T_0, has a variance of 0·88, the square of the standard deviation, 0·94, given in Table 1: 25% of this variance is removed by the correlation with ambient temperature alone, i.e. the square of the correlation coefficient, 0·500, given in Table 2. The other environmental variables account for another 5%. The total variance accounted for by all the variables listed in

Table 3 that have significant regression coefficients is just over 33% ($0{\cdot}576^2$), i.e. an additional 3% only for all the further variables.

There is, however, one very significant effect revealed by the figures in Table 3. For all the responses listed there is a substantial and consistent difference between surgeons and anaesthetists with the other staff lying somewhere between. These effects are, as is shown in Table 4, equivalent to differences of between 1·7° F. and 4·1° F. in the temperatures needed to evoke a similar comfort response in the two groups, and of as much as 12° F. in the temperature needed to produce the same

Table 3. *Coefficients of the regression equations for prediction of the subject's response. All major variables*

Variable	Response				
	T_1	T_2	T_3	T_0	P
1. Ambient temperature (° F.)	0·120	0·124	0·091	0·111	0·036
2. Water vapour pressure (mm. Hg)	0·065	0·066	0·050	0·056	0·030
3. Radiation (° F.)	0·015	0·051	0·040	0·047	0·009
4. Air movement (ft./min.)$^{\frac{1}{2}}$	NS	−0·056	NS	−0·047	NS
5. Age (for each 5 yr. over 25)	−0·048	−0·028	−0·064	−0·045	−0·041
6. Sex (female)	NS	−0·124	NS	NS	−0·263
7. Race (non-European)	NS	NS	NS	NS	−0·111
8. Build (fat, 1,—thin, 5)	NS	NS	−0·054	NS	−0·042
9. Surgeon	0·235	0·188	0·065	0·184	0·254
10. Surgical assistant	0·007	−0·017	−0·043	0·028	0·011
11. Scrubbed nurse	0·007	0·188	0·065	0·028	−0·057
12. Anaesthetist	−0·239	−0·382	−0·091	−0·236	−0·186
13. Spring (Mar.–May)	NS	0·160	NS	0·128	NS
14. Summer (June–Aug.)	NS	0·166	NS	0·117	NS
15. Autumn (Sept.–Nov.)	NS	NS	−0·166	NS	0·090
16. Stay in room (hr.)	NS	NS	NS	NS	0·042
Constant	−5·622	−5·991	−3·467	−4·929	−2·262
Multiple correlation coefficient	0·520	0·541	0·423	0·576	0·490

The form of the regression equations and the symbols used for responses are as described in Table 2.

The coefficients for the seasons were computed in terms of their differences from Winter, (December–February) as the reference period.

NS indicates that the regression coefficient concerned was not significant.

extent of visible sweating. This difference is also shown in the values for the preferred ambient temperatures calculated from the regression equations of Table 3 and given in Table 6.

Table 5 also shows the effect on the response of the other variables compared to the effect of ambient temperature. The relatively small effect of radiation, between $\frac{1}{2}$ and $\frac{1}{4}$ that of a similar ambient temperature difference, is in line with other reported observations, e.g. Koch, Jennings & Humphreys (1960) who give a relative effect of about $\frac{1}{3}$. The very small ($\frac{1}{8}$) apparent effect at head height is probably, in part, an artifact of measurement. The radiation temperature measurements for this purpose were made under the operating room lamp in the most intense part of the lamp beam. The majority of the operating lamps encountered were of the 'Hanau' type, incorporating a number of separately focussed sources, and the

surgeon generally placed his head in a gap between two of the beams. A limited number of measurements showed that approximately half the radiation effect at the position of measurement was due to the beam and half to the hot lamp casing. If the surgeon was, in fact, subject only to this latter, then the radiation temperature to which he was exposed would be one half of that recorded and the regression coefficient between this value and the response would be twice the value given in Table 3, i.e. 0·30. The value in Table 5 then becomes 4·0 in place of 8·0 and the ratio to the effect of ambient temperature $\frac{1}{4}$.

Table 4. *Equivalent thermal effect of other variables*

Change in ambient temperature, ° F., required to produce the same response in the specified class of individual as that observed in the population as a whole.

	Response				
Class character	T_1	T_2	T_3	T_0	P
Sex (female)	NS	+1·0	NS	NS	+7·3
Race (non-European)	NS	NS	NS	NS	+3·1
Surgeon	−2·0	−1·5	−0·7	−1·7	−7·1
Anaesthetist	+2·1	+3·1	+1·0	+2·1	+5·2

The responses T_1, T_2, T_3 are the scale values of the interview responses for comfort of head, trunk and feet respectively. T_0, the general comfort response, $= (T_1 + T_2 + T_3)/3$.
P is the extent of visible sweating on the forehead according to the scale described in the text. The values given have been calculated from the regression equations given in Table 3.
NS indicates that the regression coefficient concerned was not significant.

Table 5. *Equivalent thermal effect of other variables*

Change in the specific variable required to produce the same effect as 1° F. drop in ambient temperature

	Response				
Variable	T_1	T_2	T_3	T_0	P
Water vapour pressure (mm. Hg)	−1·8	−1·9	−1·8	−2·0	−1·2
Relative humidity (at 68° F.)	−10	−10	−10	−11	−7
Radiation (° F.)	−8·0	−2·5	−2·3	−2·4	−4·0
Air movement (ft./min. at 25 ft./min.)	NS	+27	NS	+30	NS
Age (yr.)	+12	+22	+7	+12	+4

For explanation, see Table 4.

Scales of warmth

Various ways of combining the variables so as to provide a single index which will predict the response of individuals under a variety of environmental conditions have been proposed (Bedford, 1964). This is especially important when the range of conditions is much greater than that encountered in our observations. For comparative purposes we have compared the adequacy of a number of these when applied to our data (Table 7). It will be seen that, except for the globe thermometer, they all show some improvement over the simple use of ambient temperature, but that they account for significantly less of the variance than the regression equations on environmental variables given in Table 2.

Table 6. *Preferred ambient temperatures (° F.), T = 3·0 (for mean observed values of the other variables)*

	T_1	T_2	T_3	T_0
Surgeons	64·8	67·1	70·0	66·1
Surgical assistants	66·7	67·4	70·5	67·5
Scrubbed nurses	66·7	67·1	70·0	67·5
Anaesthetists	68·8	71·8	71·6	69·9

T_1, T_2, T_3 are the scale values of the interview response for comfort of head, trunk and feet respectively. T_0, the general comfort response, $= (T_1 + T_2 + T_3)/3$.

The values given have been calculated from the regression equations given in Table 3 and represent the ambient temperatures at which the highest proportion of the class indicated stated that they were comfortable.

Table 7. *Comparison with other indices of thermal comfort*

Values of the correlation coefficient between the indicated response and the index.

Index	Mean value in these data (° F.)	T_1	P
Ambient temperature	72·6	0·466	0·262
Globe thermometer temperature	76·9	0·448	0·306
Equivalent temperature	72·8	0·482	0·300
Effective temperature	68·1	0·481	0·285
Singapore or Equatorial Comfort Index	67·0	0·481	0·281
Regression (Table 2)	—	0·504	0·331

T_1 is the scale value of the interview response for comfort of the head. P is the extent of visible sweating on the forehead according to the scale given in the text.

The correlation with the Singapore index was calculated from the data for surgeons, surgical assistants and scrubbed nurses only, excluding anaesthetists.

The definitions of the several indices are given in Bedford (1964).

Table 8. *Personal variation, response at head height (T_1)*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Regression	4	714	178·53	226·47
Between persons	631	870	1·38	1·75
Residual	1456	1148	0·79	—
Total	2091	2732	1·30	—

F (631, 1456) = 1·23 at the 0·1 % level of significance.

Personal consistency

On a number of occasions we interviewed the same person more than once. From this portion of the data it has been possible to compute a partition of variance between that absorbed by the regression, that due to consistent differences between individuals and the residual representing variation in the response of a single individual. The results of this calculation are given in Table 8. It will be

seen that while there are consistent personal differences these are not large. The root mean square between individuals is only 1·32 × the residual

$$[\sqrt{(1{\cdot}38/0{\cdot}79)} = \sqrt{(1{\cdot}75)} = 1{\cdot}32],$$

and the proportion of variance absorbed by the regression for repeated observations on a single individual only rises to 38% [714/(714+1148)]. Somewhat larger values, up to 50–60%, have been recorded for observations on single subjects (Davis, McMillan & Webb, 1965) but the environmental conditions were more closely standardized in these experiments.

Distribution of response

In addition to an estimate of the preferred temperature, defined above as that temperature at which the predicted comfort response has the value 3·0, it is also desirable to know the proportion of persons too hot, comfortable or too cold under

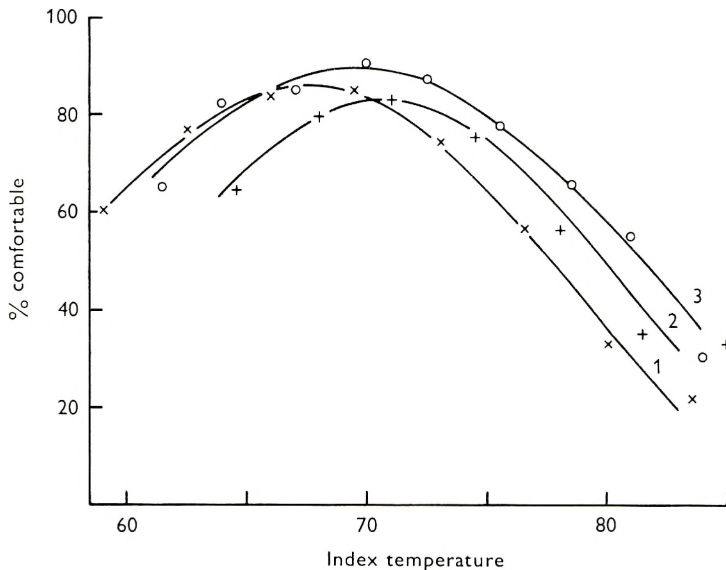

Fig. 1. Relation between index temperature and the proportion of individuals comfortable for different parts of the body. Curve 1, ×, comfortable at head. Curve 2, +, comfortable at trunk. Curve 3, ○, comfortable at feet. See text for definition of index temperature.

the various environmental conditions. For this purpose an *Index Temperature* has been defined. This is directly related to the value of the response predicted by the relevant regression equation. It is that ambient temperature in ° F. which would elicit the same predicted response in the specified population under the following standardized conditions: radiation temperature equal to the ambient temperature, relative humidity 50%, air movement 25 ft. per minute. These standard conditions have been deliberately chosen to lie near to the mean values observed so as to avoid unreal extrapolation. In making use of the term index temperature we do

not intend to add yet another to the already considerable published array of comfort indices. This form of expression is, however, convenient for the exposition of the present body of data and the method is equally applicable to other specialized situations. The index temperature as we shall use it here relates to a defined population in a particular situation, and its relation to ambient temperature and to other environmental values will depend on these specified conditions and be deducible from the relevant regression equation or equations.

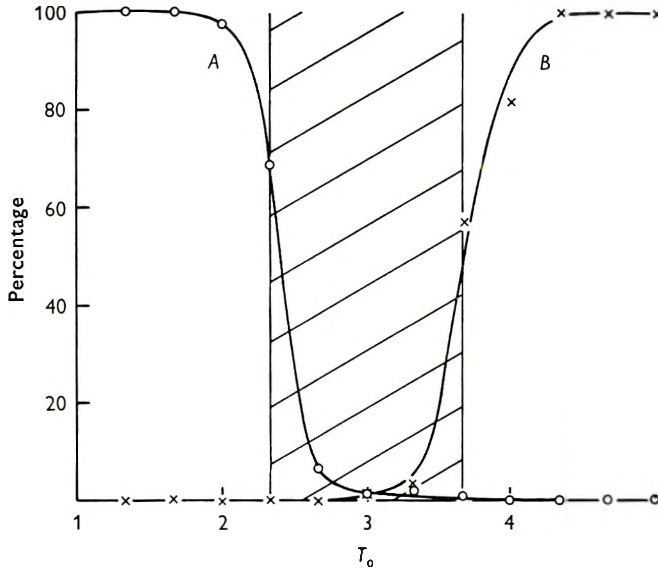

Fig. 2. Proportion of general comfort responses which included at least one 'too cold' or 'too hot' response. Curve *A* shows the proportion of the responses at a given value of T_0, the general comfort response, which included at least one 'too cold' vote, scale value 0 or 1, from either T_1, head, T_2, trunk, or T_3, feet. Curve *B* shows similarly the proportion with at least one 'too hot' vote, scale value 5 or 6. The hatched area shows the range defined as comfortable.

Figure 1 shows the proportion of individuals who stated that they were comfortable, response 2, 3 or 4, as a function of the index temperature. The proportions are shown separately for comfort of head, trunk and feet. The distributions are approximately symmetrical about the maxima and the preference for appreciably cooler temperatures about the head is clearly shown.

It is not immediately obvious how the values of the general comfort response (T_0) should be classed as comfortable, too hot or too cold. Any particular value can arise from a large number of combinations of response at head, trunk and feet, e.g. a value of 2·33 could arise from votes of 3, 3 and 2 or 1, 3 and 4 or even 1, 2 and 5. The first combination includes only votes within the comfort range, the second one 'too cold' response and the third the 'contradictory' pattern of simultaneous 'too hot' and 'too cold' responses. Each individual T_0 response was therefore examined. T_0 can only take integral or thirds values and for each possible value we determined the proportion of responses which included contributing votes, either T_1, T_2 or T_3, lying outside the comfort range of 2, 3 or 4. The results

are shown in Fig. 2. From the figure it is clear that values of T_0 of 2 or lower nearly always included at least one 'too cold' response and can therefore reasonably be taken as indicating a general response 'too cold'. Similarly, values of T_0 of 4 or more nearly always included at least one 'too hot' response and have been taken as defining a general response of 'too hot'. The range 2·33–3·66 inclusive has therefore been taken as the comfortable range. At the limits of this range there is little more than a 50% chance of any individual response lying outside the values 2, 3 and 4. Only 7 out of the more than 2500 values involved simultaneous 'too hot' and 'too cold' responses and these have been excluded from the analysis.

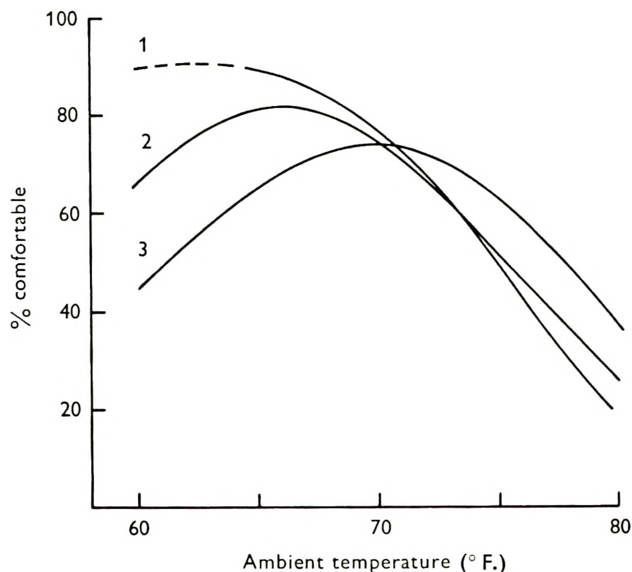

Fig. 3. Proportion of individuals comfortable, general comfort response T_0, at various ambient temperatures. Curve 1 refers to surgeons. Curve 2 refers to surgical assistants and scrubbed nurses. Curve 3 refers to anaesthetists.

The distribution of values of T_0, grouped into 'too cold', comfortable and 'too hot' in this way, is shown in Figs. 3 and 4. Figure 3 shows the proportion comfortable for surgeons, anaesthetists and other table staff, considered separately, as a function of simple ambient temperature. In order to obtain these curves the probits of the proportions 'too hot' and 'too cold' were first plotted against the temperature. This transformation gives a straight line relationship. Smoothed estimates of the proportions 'too hot' and 'too cold' were then obtained from these lines and the proportion 'comfortable' obtained by difference, i.e. those neither 'too hot' nor 'too cold'. The relatively low temperature preferred by surgeons compared with the anaesthetists is apparent. In Fig. 4 the same data for all categories of table staff combined are shown as a function of index temperature. The proportions 'too hot' and 'too cold' have been plotted as probits while the proportion comfortable is given as a simple percentage. The line for the proportion too hot can be used to estimate the index temperature required to be attainable in order to ensure that no more than a specified proportion of the potential occupants

will feel too hot. For no more than 5 % of operating room table staff this is 67° F. and for no more than 1 % 63·5° F. In specifying the environmental conditions that should be attainable in an operating room, where the comfort of only one or a few individuals is involved, these values are more important than the value at which the largest proportion of persons is comfortable.

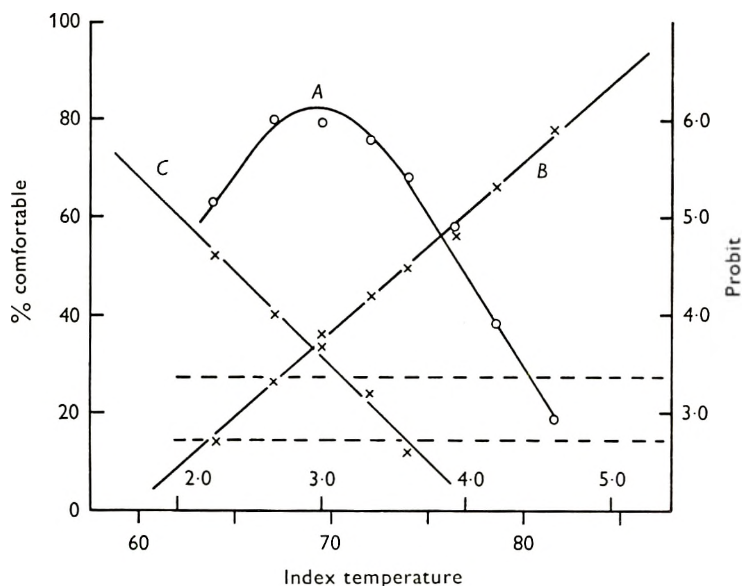

Fig. 4. Proportion of individuals comfortable, general comfort response T_0, at various index temperatures together with the proportion 'too hot' and 'too cold'. Curve A shows the percentage comfortable. (L.H. scale). Curve B shows the probit of the percentage 'too hot'. (R.H. scale). Curve C shows the probit of the percentage 'too cold' (R.H. scale). The figures above the index temperature scale give the equivalent predicted values of T_0. Curve A has been calculated from the probit lines B and C. Definition of the comfort range boundaries is given in the text. The broken lines indicate the 1 and 5 % probit levels (R.H. scale).

Sweating

There is, of course, no preferred level of sweating and these data have been dealt with somewhat differently. The regression equations show that observed sweating is a sensitive index of personal difference and responds to changes in the environmental variables in a similar way to the comfort response. This is of considerable interest since, unlike the comfort response, it is an objective estimate of response to thermal conditions. It is, of course, one-sided. Conditions that are too hot evoke a response but not those that are too cold. The fact that conditions in the operating rooms we observed were much more often too hot than too cold undoubtedly contributes to the regular behaviour of this response. A limited number of actual measurements of the amount of sweat produced were made on the upper arm and on the forehead. These will be reported in more detail elsewhere. They confirmed, however, the opinion that a large part of the sweating observed in the operating room, although related to the thermal environment, is also substantially a response to stress (Kuno, 1956). This is shown by the much higher rate of sweat production

by the surgeons and the much greater rate observed on the forehead compared to the upper arm. In Fig. 5 the proportion of individuals who were visibly sweating to each of the four recorded levels is shown plotted against index temperature.

By comparison of the regression coefficient for temperature with the status coefficients which are given for visible sweating, P in Table 3, it will be seen, as is also shown in part in Table 4, that the equivalent index temperatures for surgeons are 7° lower, but for scrubbed nurses are 2° higher and for anaesthetists are 5° higher, than the average for all table staff. Under the standard conditions of 50% relative humidity, 25 ft. per minute air movement and no excess radiant temperature, this means that, while 50% of all table staff were visibly sweating at an

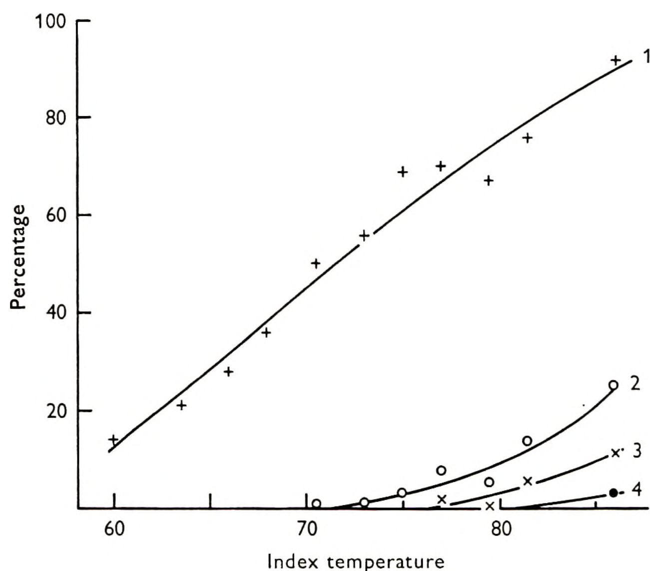

Fig. 5. Proportion of individuals sweating. Curve 1, proportion visibly sweating. Curve 2, proportion with beads of sweat on the forehead. Curve 3, proportion from whose forehead sweat had to be wiped away. Curve 4, proportion whose clothing was wetted through with sweat.

ambient temperature of 72° F., 50% of surgeons were sweating at only 65° F. A temperature of 77° F., however, had to be reached before 50% of the anaesthetists were sweating. Similarly, while 80% of all staff were visibly sweating at a temperature of 82° F. under the standard conditions, this proportion of surgeons was visibly sweating under the same conditions at only 75° F., at which temperature 5% of the surgeons were sweating to such an extent (grade 3) that the accumulated sweat had to be wiped from their foreheads.

Although the effect of the radiant heat from the operating lamp, to which the surgeons are exposed, is to lower still further the ambient temperature at which surgeons sweat, the reduction is only slight, as is reflected in the small value of the corresponding regression coefficient in Table 3.

Other factors affecting comfort

The analysis of the effect of the large number of other factors that were recorded produced only a limited number of results which added anything to those already described. In particular, the regression on interview responses such as stuffiness, air movement, noise, etc., were not such as to contribute further to our understanding of the problems of thermal comfort. A few of the factors which were investigated as described earlier, by examining the differences between the responses predicted by the regression equations given in Tables 2 and 3 and those actually recorded did, however, show some interesting relationships. These are shown in

Table 9. *The response to some environmental circumstances*

Circumstance	Change in ambient temperature, ° F., which would produce a similar response to that caused by the specified circumstance	
	Comfort at head (T_1)	Sweating (P)
1. Tense atmosphere	0·9	2·7
2. Major operation	NS	1·1
3. Mistake in procedure	−0·6	NS
4. 50 % more entries and exits per hour than average	NS	0·8
5. 50 % increase in movement	NS	−1·1
6. 5 phons increase in noise	NS	−1·4

The average number of exits and entries from and into an operating room was 122/hr. Movement in the operating room was recorded in terms of the number of movements between a limited number of arbitrarily chosen focal points in the room. Noise here represents that level which was reached or exceeded for 20 % of the record. This level averaged approximately 60 phons.

NS indicates that no significant regression coefficient was obtained in the analysis.

The atmosphere in the operating room was recorded as: 0, tense; 1, normal; 2, relaxed. Operations were classified as average, major or minor.

Table 9, in terms of their equivalent thermal effect. There was more sweating and the staff felt hotter when the 'atmosphere' in the operating room was tense and when there were more frequent exits from and entries to the operating room. These two conditions tended to be associated with each other. On the other hand, when there was more movement in the room and when the noise level was raised, usually by talking, the more relaxed atmosphere implied by these activities was associated with less sweating. The response to 'mistakes' or to the carrying out of a major operation was not, however, consistent. These observations emphasize the subjective elements in comfort and sweating.

An increase in the variability of the velocity of air movement was also shown to influence the perception of comfort for the head. The average value of the mean deviation of the individual measurements of air velocity about their mean value on any occasion was 4·3 ft./min. (The individual readings had been taken in groups of two at 5 sec. intervals and the time constant of the recording instrument was

of the same order, about 5 sec.). A doubling of this, i.e. to a mean deviation of 8·6 ft./min., was accompanied by a fall in the scale value of the response for comfort for the head equivalent to a drop of 0·9° F. in ambient temperature.

No significant effect on response could be attributed to the temperatures in the ancillary rooms of the operating suite, nor to the average outdoor temperature during the previous fortnight.

Index temperature

A substantial proportion of the results discussed have been related to a so-called index temperature. In using the regression equations to convert this to actual environmental conditions for a given class or group of operating room staff,

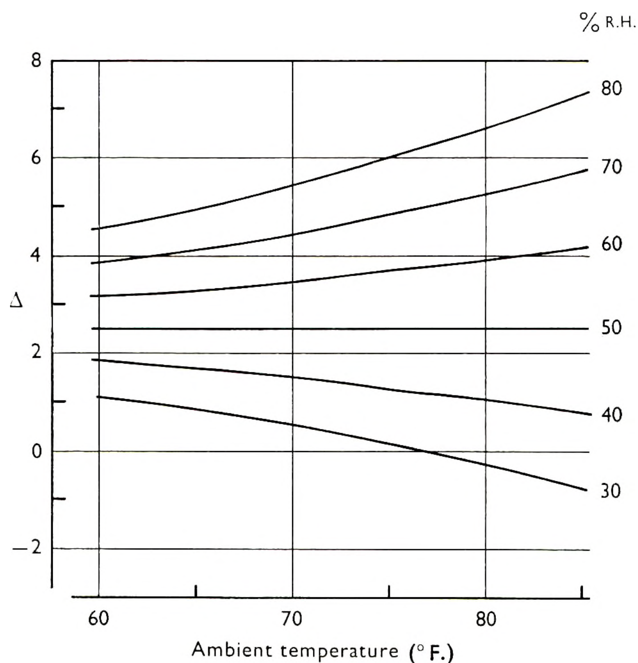

Fig. 6. Relation between index and ambient temperature for the general comfort response, T_0. The value of the index temperature is given by: index temperature = ambient temperature $+\Delta-\frac{1}{2}\sqrt{v}+\frac{1}{2}R+A$, where Δ is read from the figure, v is the air velocity in feet per minute, R is the excess of radiation temperature over ambient air temperature in ° F. and A is a constant which is zero for the reference population (average of all operating room table staff) and takes the value 1·6 for surgeons, 0·25 for surgical assistants and scrubbed nurses and −2·1 for anaesthetists. All temperatures are in ° F.

it is important to remember that while the index temperature is defined in terms of 50% relative humidity, the regression equations are given in terms of water vapour pressure (mm. Hg). Regression equations in terms of relative humidity would be more complex in view of the interaction between relative humidity and temperature referred to in the section on analysis. In order to facilitate the conversion, Fig. 6 has been drawn to show the relationship between index temperature and ambient temperature for the general comfort response, T_0.

The effect of humidity on the comfort responses at head, trunk and feet, T_1, T_2 and T_3, is practically identical with that on T_0 so that the figure may also be used for calculations relating to these. The coefficients of the effects of air movement, $\sqrt{v}$, thermal radiation, R, and individual function, the constant A, will, however, differ and must be estimated from the regression equations given in Table 3 by dividing the appropriate coefficient by the coefficient for ambient temperature in the same regression equation.

DISCUSSION

This investigation has established that an index temperature, as defined earlier, of 69° F. is that at which the highest proportion of operating room staff in Britain are comfortable. This figure is not, however, in itself of much significance since there is, in the first place, a substantial difference in the temperature preferences of surgeons, anaesthetists and other table staff. Equally important, the requirements for specifying a comfortable thermal environment in an operating room differ from those in offices, factories and those other environments to which most previous thermal comfort studies have been directed. These environments are occupied simultaneously by a large number of individuals and the problem is to specify those conditions which will be comfortable to the highest possible proportion of these. The operating room is occupied by only a small number of persons at any one time, and the comfort of only one or a few of these, the surgeons, may be considered the most important. In these circumstances, it is desirable to specify those thermal conditions which must be capable of being attained in order that no more than a small proportion of these persons need be uncomfortable. In the operating room environment sufficient warmth is no problem, so that we need to specify how low a temperature must be attainable. If we can allow no more than 5% (1 in 20) of individuals to be too hot, Fig. 4 shows that the index temperature must be capable of being held down to no more than 67° F. but if only 1 in 100 may be too hot then the figure is probably about 63·5° F. For surgeons the equivalent index temperatures are rather more than 1·5° lower than for the average of all staff, and in addition it is necessary to make some allowance for radiation from the operating lamp, probably about 1°. These two effects together amount to about 2·5°, so that for no more than 1 surgeon in 20 to be uncomfortably hot an equivalent index temperature of 64·5° F. must not be exceeded, i.e. 64·5° F. at 50% relative humidity and 25 ft./min. air movement. The index temperature should probably not exceed 61° F. if no more than 1 in 100 is to be uncomfortably hot.

Surgical assistants and scrubbed nurses are also exposed to thermal radiation from the operating room lamp and will generally prefer temperatures about 1° F. higher than the principal surgeon. This difference is not large enough to be significant. Anaesthetists, however, are usually more remote from the operating lamp and in addition prefer temperatures nearly 2° F. above the average for all staff, i.e. an index temperature of about 71° F., corresponding to an ambient temperature about 4° F. warmer than that preferred by most surgeons.

At the relatively low temperatures needed to prevent the more heat-sensitive surgeons from feeling too hot a substantial proportion of the other table staff will

feel too cold. At 64·5° F. ambient temperature, for example, with average radiant heat conditions and 25 ft./min. air movement at 50% relative humidity, over one-third of surgical assistants and scrubbed nurses and nearly one-half of the anaesthetists would have felt too cold. At 61° F. the proportions would be nearly two-thirds and three-quarters respectively. The only way of reconciling these differences would seem to be for the anaesthetists and perhaps some others of the table staff to wear rather warmer clothing in operating rooms which can be kept down to the surgeon's preferred temperature. Recent work on the dissemination of bacteria from various forms of clothing shows that, even if the additional clothing were not sterile, it should not lead to any increased risk of infection for the patient (Bethune, Blowers, Parker & Pask, 1965).

It is not possible to estimate how often regulation of temperature conditions by the anaesthetist, who can do this readily where open controls are provided, may have contributed to complaints of overheating by surgeons. It is, however, probably just coincidence that the mean temperature recorded during these observations, approximately 72° F., lies so close to that preferred by most anaesthetists (see Fig. 3).

The effect of all the other variables observed was small in comparison with that of ambient temperature. In particular the effect of air movement was slight and not always significant. This is perhaps related to the very small skin areas exposed. The observed extent of visible sweating provides a good objective index of thermal discomfort in too hot conditions. This too is not greatly affected by environmental conditions other than ambient temperature. The effect of race and sex is however more pronounced on visible sweating (Table 4) than on the expression of comfort. Almost all the sex difference, however, depends on observations on anaesthetists, the only class which included a substantial proportion of both sexes. The much greater extent of sweating among surgeons is almost certainly a reflexion of nervous tension. Observations on the actual amount of sweat produced showed that this was almost entirely confined to the forehead. Upper arm sweating was very slight under the conditions investigated.

Although the other environmental variables exert individually only small effects their combined influence is not necessarily negligible. Thus, a reduction of 10% in relative humidity and 2° F. in the radiation temperature, accompanied by an increase of 15 ft./min. in the air velocity in the operating room, with some increase in the variability of this rate of air movement, will usually be equivalent in its effect on comfort and sweating to a drop of between 2 and 3° F. in the ambient temperature. This calculation assumes that the initial conditions are close to the average values of Table 1.

The figures given above define the requirements for thermal comfort of surgeons working in the British Isles under present-day surgical conditions. If the well-being of the patient calls for higher temperatures, or inadequate plant makes these inevitable, some amelioration of the consequent discomfort to the surgeons may be achieved by other methods. Surgical clothing, including the wearing of masks, undoubtedly imposes a thermal load on the wearer. The extent of this does not appear to be known, particularly the extent to which masks promote re-breathing

and warm and humidify the inspired air. Modifications in design may be able to lighten this thermal load, although the bacteriological requirement for closely woven materials impermeable to small particles (Bernard, Speers, O'Grady & Shooter, 1965; Blowers & McCluskey, 1965) does not help. Charnley (1965) has approached the problem in a more radical way by furnishing the surgeon with an independent piped air supply and extract to the inside of his suit and mask.

This investigation largely substantiates the claim that conditions in British operating rooms often reach temperatures above those at which surgeons are comfortable. The average temperature observed in this series was over 72° F., nearly 6° F. higher than the value most generally preferred by surgeons. A temperature of 64·5° F., which seems to be necessary if no more than 5% of surgeons are to be uncomfortably hot, cannot be attained without some degree of cooling over part of the year, even if extraneous heat is kept to a minimum.

SUMMARY

Visits have been made to thirty operating suites in the British Isles. Each suite was visited three times at approximately 4-month intervals and observations made on an average of five half-day working sessions on each occasion. Measurements were made of air temperature, humidity, air movement and radiation temperature and many details of the suites and working conditions recorded. At suitable intervals the operating room staff were questioned as to their feelings of thermal comfort using Bedford's 7-point scale. Over 2500 sets of replies were obtained. Visible sweating was also noted.

The effect on comfort and the extent of visible sweating of the many items recorded was then explored by means of a sequential multiple regression analysis. Although air temperature was by far the most important factor affecting thermal comfort, all the variables named above exerted a significant effect. In addition, a number of other conditions including age, sex and race produced minor differences.

Surgeons and anaesthetists were found to differ from other staff in their thermal preferences, the surgeons liking a cooler and the anaesthetists a warmer environment. Although most surgeons were comfortable at temperatures around 66·5° F. (19° C.), at 50% relative humidity and 25 ft./min. air movement with the average amount of thermal radiation from the operating room lamp, it would be necessary to keep the temperature down to 64·5° F. (18° C.) if no more than one surgeon in twenty was to be uncomfortably hot. At this temperature nearly half the anaesthetists, who mostly preferred temperatures around 71° F. (21·5° C.), would feel too cold. Variation in the clothing worn by different staff members seems to be the only way of resolving this difficulty.

The average temperature in the operating rooms visited was over 72° F. (22° C.), and 75° F. (24° C.) was exceeded on about 25% of occasions.

We should like to thank all those, operating room staff and others, whose help and collaboration made this investigation possible.

This work includes part of the material contained in a thesis to be submitted by one of us (D.P.W.) for the Ph.D degee of the University of London.

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Serological tests for the detection of antibodies to *Mycoplasma gallisepticum* in chickens and turkeys

By F. T. W. JORDAN and P. KULASEGARAM
Poultry Section, Department of Veterinary Preventive Medicine, University of Liverpool Veterinary Field Station, Neston, Wirral, Cheshire, England

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INTRODUCTION

The relative sensitivity and specificity of the various tests used in the examination of avian sera for antibodies to *Mycoplasma gallisepticum* have been examined by only a few workers. Jungherr, Luginbuhl, Tourtellotte & Burr (1955) compared the whole blood (WB), slide agglutination (SA), tube agglutination (TA) and haemagglutination inhibition (HI) tests on sera from chickens and turkeys naturally infected with *M. gallisepticum*. Hofstad (1957) compared the SA, TA and HI tests on the sera of naturally infected turkeys. Tests were conducted at irregular intervals for a period of 12–21 months, commencing with some birds 2 months after the disease outbreak. Adler (1958) compared the SA and TA tests on the sera of seventy naturally infected turkeys 15 weeks after infection and the sera of sixty-two of them after 27 weeks. Rhoades, Kelton & Heddleston (1965) compared the HI and TA tests on the sera of twenty-four adult turkeys experimentally infected with *M. gallisepticum* and examined their sera at weekly intervals for 11 weeks. Adler & DaMassa (1964) compared the antiglobulin test with the TA test on the sera of five chickens infected experimentally with *M. gallisepticum*, and bled at irregular intervals for 31 days. Baharsefat & Adler (1965) compared the TA, HI and antiglobulin tests on the sera of nine chickens and two turkeys experimentally infected with *M. gallisepticum*. Sera were collected at irregular intervals for 30 days from chickens and 63 days from turkeys.

Leach & Blaxland (1966) showed that the method of preparation of the reactants and the method by which a test is performed will influence its sensitivity and suggested that these factors must be considered when tests are compared, especially when undertaken in different laboratories.

Because it was considered worth while repeating some of these comparative tests and including such other tests as indirect complement fixation (ICF), metabolic inhibition (MI), and gel precipitation (P), giving details of reactants and methods, a series of experiments were undertaken in which sera from chickens and turkeys infected intratracheally with the A514 strain of *M. gallisepticum* were examined.

MATERIALS AND METHODS

Chickens. Brown and White Leghorn chickens were hatched at the Laboratory from eggs from flocks free from the common respiratory pathogens and from resistance-inducing factor (RIF), and were reared in isolation. Cockerels were infected at 4–8 months old, and housed individually in cages; the birds in the different experiments were kept in separate houses.

Turkeys. Broad Breasted White turkeys from a mycoplasma-free flock were reared from 1 day old in isolation. Turkeys of both sexes were infected at 5–10 months of age, and birds in the different experiments were kept in different houses.

Rabbits. English adults were used.

Mycoplasma strain. The A514 strain of *M. gallisepticum* originally obtained from Dr Chu at Cambridge was used. It had undergone numerous passages in artificial medium.

Brucella broth medium (B.B.M.). Brucella broth* medium was prepared by reconstituting the broth powder following the manufacturer's instructions with the addition of 15% heat-inactivated swine serum, 1% of 1/20 solution of thallous acetate, 50,000 units penicillin (crystalline sodium salt) per 100 ml. and 1% Andrade's indicator.

The pH of the broth was checked and in our experience was always about 7·4. It was filtered through a Seitz EK filter, stored at 0–5° C. in a refrigerator and used within a month of preparation.

Chicken infusion medium (C.I.M.). A modification of the method of Hofstad & Doerr (1956) was used. To prepare 100 ml., 50 g. of chicken meat removed from the breast and legs and liver and heart was cut into small pieces or minced, added to 100 ml. of glass-distilled water and kept overnight in the refrigerator. Next morning it was boiled for 20 min. in a water-bath and filtered through No. 1 Whatman filter paper to remove gross particles. To 80 ml. of this infusion were added 20 ml. of heat-inactivated chicken serum, 1·0 ml. of a 1/20 solution of thallous acetate, 50,000 units of penicillin, 1·0 ml. of Andrade's indicator, 0·5 g. sodium chloride, 0·2 g. glucose (bacteriological), and 1·0 ml. of 1% solution of nicotinamide-adenine dinucleotide. The pH was adjusted to 7·4 with 10% sodium hydroxide, and it was filtered through a Seitz EK filter and stored at 0–5° C. in the refrigerator. It was used within 1 month of preparation.

Turkey infusion medium (T.I.M.). Prepared as C.I.M. except that turkey meat and serum replaced chicken meat and serum respectively.

Rabbit infusion medium (R.I.M.). Prepared as C.I.M. except that rabbit meat and serum replaced chicken meat and serum respectively.

All media supported the growth of the A514 strain and when the proportion of inoculum at log phase to medium was 1:50, the culture usually attained log phase in 24 hr.

Sera. Sera from experimental birds were removed from the clot within 24 hr. of bleeding, inactivated by heating at 56° C. for half an hour, and stored at 0–5° C. They were examined before storage or within 3 months of it.

* Albimi Labs. Inc., Brooklyn, New York.

Serological tests

Agglutination tests

Three agglutination tests were undertaken: WB, SA, and TA tests. Antigen for these tests was prepared from the A514 strain grown in B.B.M. to log phase, tested for sterility, washed three times in phosphate-buffered saline of pH 6·8 (PBS) with 0·25 % phenol and standardized to an opacity of 15 times No. 4 'Wellcome' tube. Each fresh batch of antigen was also compared with a previous batch for titre and speed of agglutination by the SA test on known positive sera. If less sensitive than the previous batch it was discarded; none were found more sensitive.

Unstained antigen was used for the SA and TA tests, while the antigen for the WB test was the same antigen stained with aqueous crystal violet to give a final concentration of 1/10,000. It was stored at 0–5° C.

Whole blood test. Stained antigen in 0·04 ml. volumes was pipetted on to a white tile and an equal quantity of blood from the wing vein was added with a standard loop. They were mixed and examined after 2 min. for chicken blood and 3 min. for turkey blood. The temperature of the plate was kept at approximately 65° F. (16·5° C.).

Slide agglutination test. Antigen and serum were mixed in equal volumes (0·02 ml.) on a glass slide and read after 2 min. for chicken sera and 3 min. for turkey sera. A known positive and negative serum were included.

The antigen for the WB and SA tests was periodically agitated between tests to keep the organisms in a uniform suspension.

Tube agglutination test. The antigen was diluted 1/15 with PBS for chicken sera and 1/20 for turkey sera. Doubling dilutions of the sera were made from 1/5 in PBS in agglutination tubes. To 0·5 ml. amounts of each of these dilutions was added an equal volume of the antigen. Undiluted serum was also tested. Controls consisted of a positive and negative serum and a tube containing 0·5 ml. of PBS and an equal volume of antigen. The tubes were shaken, incubated at 56° C. for 2 hr. and kept at 0–5° C. overnight and read. The TA test on hyperimmune rabbit sera was conducted as for chicken sera.

Haemagglutination inhibition test

The antigen for the HI test was prepared as described for the agglutination tests except that the organisms were washed in PBS without phenol, and finally suspended in 50 % glycerol–saline to give a final concentration of organisms 100 times that of the original culture. It was stored at −27° C. and used within 6 months of preparation.

A modification of the method of Roberts (1964*a*) was used for the standardization of antigen and for the test itself. A 0·75 % suspension of chicken erythrocytes for chicken sera and 0·75 % suspension of turkey erythrocytes for turkey sera in PBS was used. All reactants were used in volumes of 0·2 ml. In the standardization of antigen 100 % haemagglutination (HA) was taken as the end-point of reaction, and at titre one haemagglutinating dose of mycoplasma was contained in 0·2 ml. Four haemagglutinating doses of antigen standardized in this way were used in the HI test, in which complete inhibition of haemagglutination was the end-point. Initial

work showed that non-specific inhibition which occurred with 4 HA units of antigen standardized to 50% HA was prevented by using 4 units of antigen standardized to 100% HA.

For the HI test serial doubling dilutions of serum were prepared from 1/5 in PBS. Undiluted serum was also tested. Antigen and cell controls were included and readings were taken when settling had occurred in the cell control well.

The indirect complement fixation test

In addition to the titration of complement and haemolytic serum, rabbit antiserum prepared against *M. gallisepticum* was titrated in a CF test against *M. gallisepticum* antigen to determine optimal proportions. The antigen was prepared as for the agglutination test but subjected to ultrasonic disintegration.

The ICF test was performed as a 3-day test. On the first day a twofold series of dilutions* was made of each serum, from 1/1 to 1/1024, and 0·1 ml. volumes were transferred to wells of a Perspex tray. Each well then received 0·1 ml. of *M. gallisepticum* antigen containing 1 (OPD) unit. The tray was gently shaken and kept at 0–5° C. overnight. On the second day 0·1 ml. rabbit serum containing 2 (OPD) units was added to each well, and then 0·1 ml. containing 2 units of complement. The tray was shaken and kept at 0–5° C. overnight. On the third day the tray was removed and placed in the incubator at 37° C. for 30 min. To each well was then added 0·1 ml. of sensitized 2% sheep red blood cell suspension, the tray was re-incubated for 30 min., shaken occasionally, allowed to stand at room temperature for 1 hr., and readings were taken. The highest dilution of the serum associated with 50% lysis of the red blood cells was considered to be the titre of the serum.

The test included controls for the amount of complement, for non-specific lysis of red cells by diluent, for optimal proportion of antigen and rabbit serum, and anticomplementary activity of the antigen and rabbit serum.

Metabolic inhibition test

Doubling serial dilutions of the serum from 1/2 were made in B.B.M. with 0·002% phenol red as indicator; 0·1 ml. quantities of each dilution of serum and of undiluted serum were transferred to tubes containing 0·5 ml. of B.B.M.; and then 0·2 ml. of a log phase culture of *M. gallisepticum* diluted 1/10 in B.B.M. was added to each tube. The controls for the test included a tube containing 0·6 ml. of B.B.M. inoculated with 0·2 ml. of diluted culture to determine the first appearance of colour change and another tube containing 0·8 ml. of B.B.M. for matching colour. The tubes were sealed with sellotape and incubated at 37° C. until the first colour change was seen in the control. The highest dilution of serum which prevented the colour change at this time was taken as the metabolic inhibition titre of the serum.

Precipitation test

Precipitation tests were undertaken by the double diffusion agar gel technique carried out in Petri dishes. The agar gel contained 1·5% 'Special Agar Noble'

* The dilent used in the ICFtest was prepared from Barbitone, CFT tablets as recommended by the manufacturer (Oxoid Ltd).

(Difco), 8 % sodium chloride (1 % when testing rabbit serum) and 1 % sodium azide. Bentonite (1·5 %) and Hyflo-Super cel* (1·5 %) were added for clarification (Feinberg, 1956). Wells were cut in the agar as shown in Fig. 1. Two adjacent wells

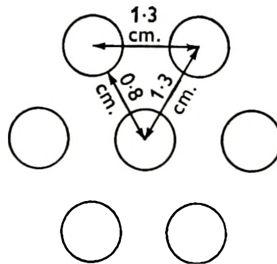

Fig. 1. Size and arrangement of wells in the agar-gel precipitation test.

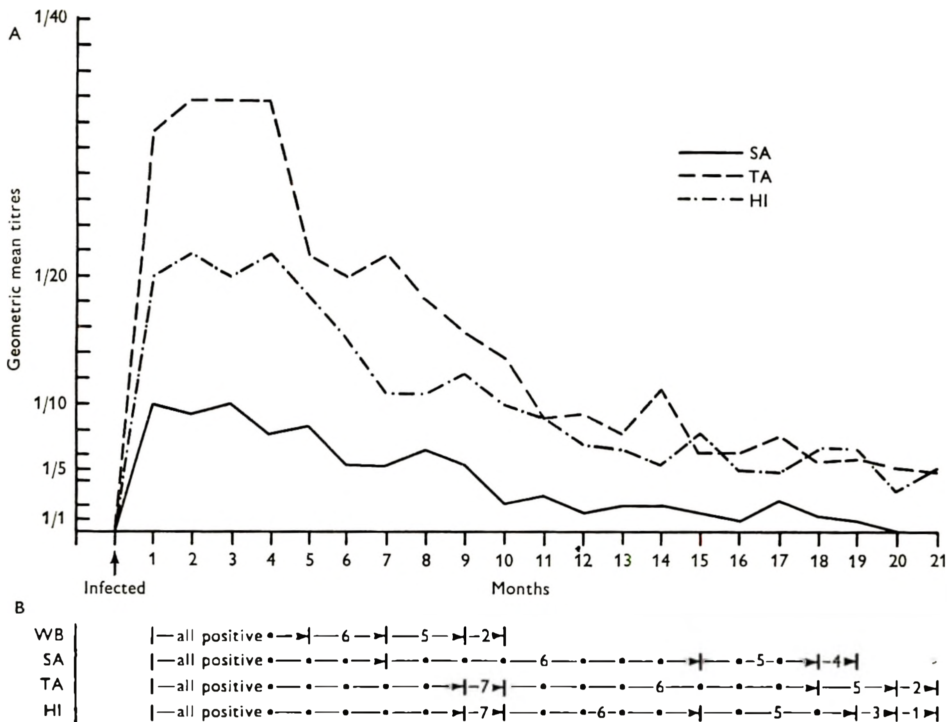

Fig. 2. A. The geometric mean titres of eight chickens inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors shown by WB, SA, TA and HI tests.

were used for each serum sample, the plates were incubated at 37° C. in a moist atmosphere for 5 days and examined daily. Antigens were prepared by concentrating a culture in B.B.M. 25, 50 and 100 times and resuspending the organisms in the supernatant. Only undiluted serum was tested.

* Johns-Manville, U.S.A.

RESULTS

Experiment 1

Eight 5-month-old Brown Leghorn cockerels were inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* grown in B.B.M. containing 10^9 viable organisms per ml. At the time of inoculation and monthly for 21 months, the chickens were bled, the blood examined by the WB test and the sera by the SA, TA and HI tests.

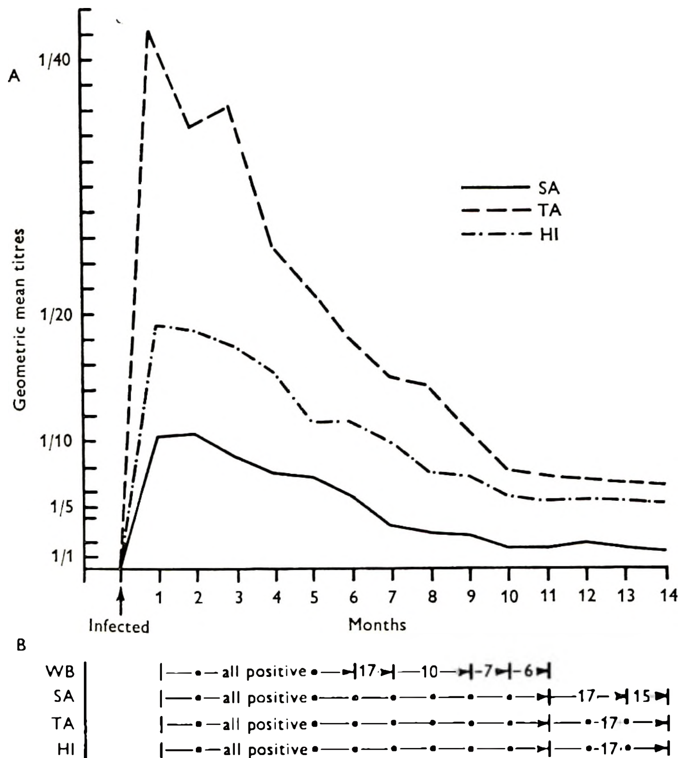

Fig. 3. A. The geometric mean titres of twenty-two chickens inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors shown by WB, SA, TA and HI tests.

The geometric mean titres of the sera are shown in Fig. 2. At the time of inoculation all sera were negative by the above tests, but 1 month after infection the sera of all birds was positive by all tests and peak titres were obtained during the first 4 or 5 months.

A similar pattern was shown by all the tests. At first all the birds gave positive reactions but after about 5 months there was a gradual decline in the number with positive sera. The largest number of positive reactors at all periods after infection were obtained with the TA and HI tests while the least number were obtained by the WB test. Highest titres were obtained with the TA test, which were approximately one dilution higher than the HI titres and two dilutions higher than the SA

titres during the first 5 months. After this there was little difference between TA and HI titres, both of which were about one dilution higher than the SA titres. The titre of the serum from a bird was occasionally found to be higher at one test than at the previous test. However, this was never more than one dilution by any of the tests and there was not a similar pattern of fluctuation shown by all three tests on the same serum. Once a bird had shown a negative reaction by a test it never subsequently showed a positive reaction by the same test.

Experiment 2

Experiment 1 was repeated with twenty-two 1-year-old Brown Leghorn cockerels which were inoculated intratracheally with 3 ml. of *M. gallisepticum* culture grown in C.I.M. containing 10^8 viable organisms per ml. At the time of inoculation and at monthly intervals for 11 months they were bled and the blood and sera examined. Five were now removed for another purpose and the remaining seventeen tested as before for a further 3 months.

The results are shown in Fig. 3 and closely parallel those obtained in Expt. 1.

The highest titres were obtained with the TA test, followed by the HI test, and all seventeen birds were positive reactors to these tests when the experiment ended. Lower titres were obtained with the SA test and two of the seventeen birds were negative at 14 months.

After 6 months fewer reactors were detected by the WB than by the other tests and none after 11 months.

Experiment 3

In order to study the antibody response to infection within the first month of inoculation and to compare additional serological tests, twenty-six 10-month-old chickens were inoculated intratracheally with 3 ml. of *M. gallisepticum* culture grown in C.I.M. containing 10^{10} viable organisms per ml.

In addition to agglutination and HI tests the sera were examined by the ICF, MI and precipitation tests. The chickens were bled at the time of inoculation and every 2 days for 10 days, when they were divided arbitrarily into two groups, the groups being bled alternately, so that individual birds were bled every 4 days. This was continued for a month, after which all the birds were bled at monthly intervals until the termination of the experiment after 5 months. One bird died 3 months after inoculation.

The results are shown in Fig. 4. Positive titres were obtained from some sera by all except the WB and P tests by as early as the 4th day and all birds were positive to all the tests except the precipitation test by the 8th day. By the 10th day maximum titres were attained; the highest with the ICF test and the lowest with the MI test. The same pattern was shown by agglutination and HI tests as in earlier experiments. At the end of the first month titres obtained with the ICF test had fallen below those seen with the TA test and at 2 months twenty-three birds had positive sera, but none were positive after 4 months, whereas sera tested by the TA and HI tests were positive throughout. All the sera were anticomplementary up to a dilution of 1/8.

By the MI test low titres were obtained with all the sera, the highest being 1/8, 6–12 days after inoculation. All were negative after 4 months.

Sera from only five of the twenty-six chickens gave a positive precipitation reaction. All five showed positive reactions with sera taken on the tenth day and some on the eighth and twelfth days, as shown in Table 1.

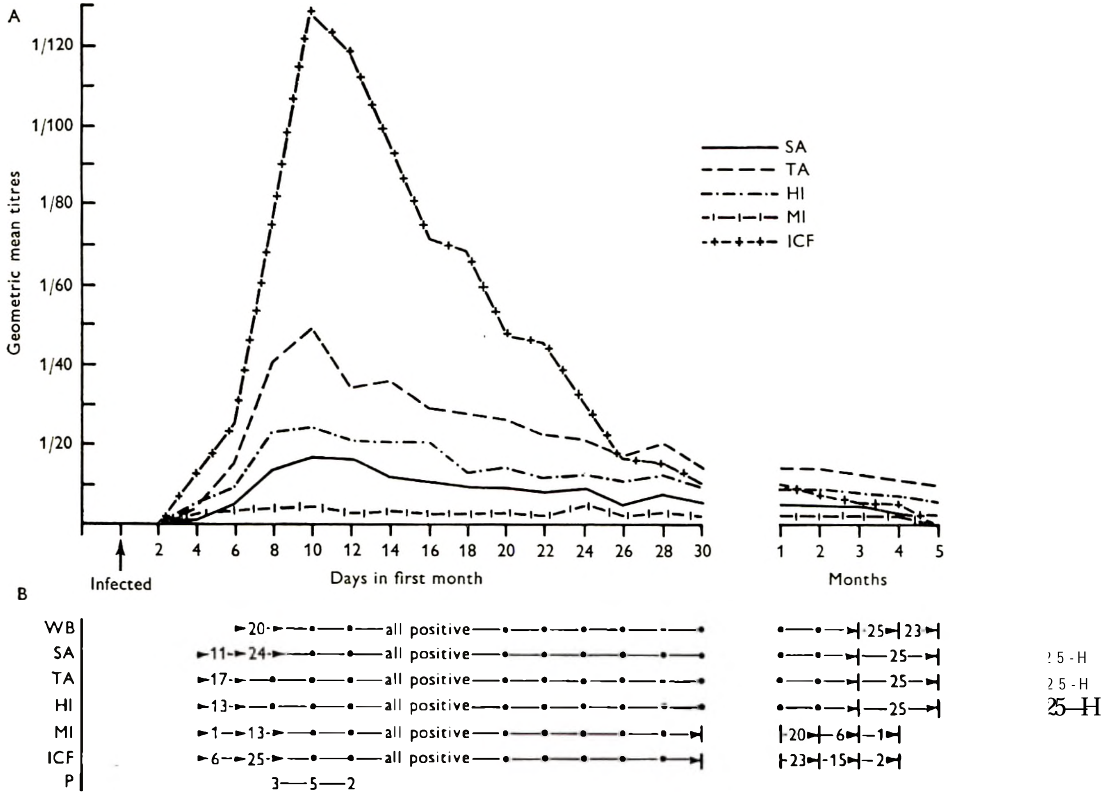

Fig. 4. A. Geometric mean titres of twenty-six chickens inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors by the WB, SA, TA, HI, MI, ICF and P tests.

Table 1. *Time of appearance of positive precipitation reactions in five chickens in Expt.* 3

Chicken no.	Days after inoculation 8	10	12
1	−	+	+
2	+	+	+
3	−	+	−
4	+	+	−
5	+	+	−

Experiment 4

Twenty-one 5-month-old turkeys were inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* in T.I.M. containing 10^9 viable organisms per ml. They

were bled every other day for 1 month and monthly for a further 2 months, and the blood and sera examined.

The pattern of results with the agglutination, HI and MI tests (Fig. 5) was similar to that obtained with chicken sera except that peak titres were lower and the period during which positive reactions were obtained was shorter. The highest titre, 1/40, was obtained with the TA test on the sera of four birds, while titres of 1/20 were obtained with the HI and SA tests on the sera of eight and two birds

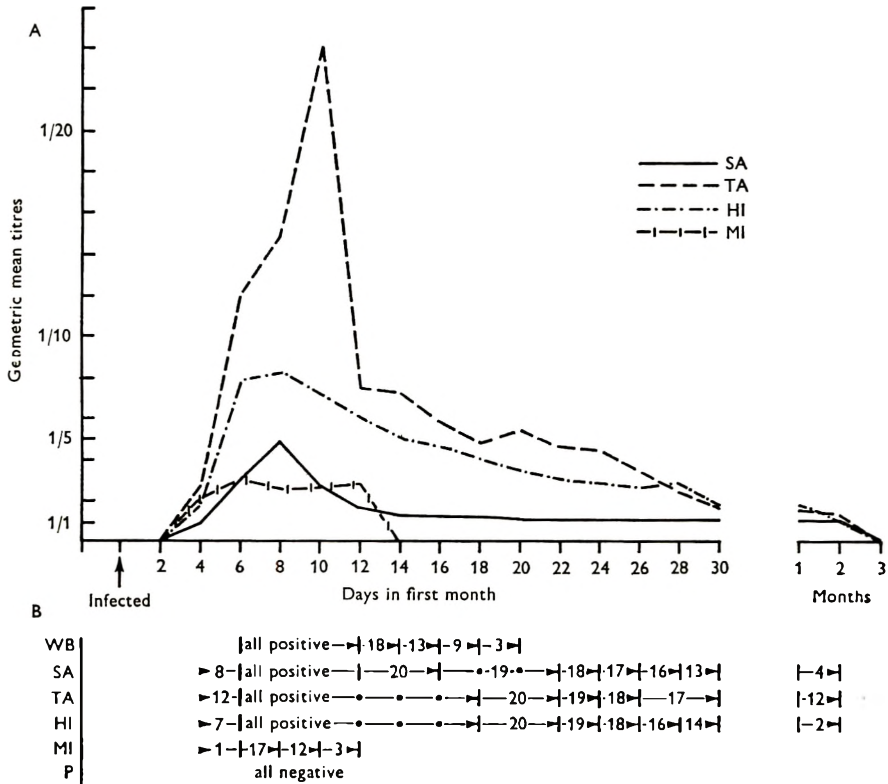

Fig. 5. A. The geometric mean titres of twenty-one turkeys inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors by the WB, SA, TA, HI, MI and P tests.

respectively. By all tests the highest titres were observed about 10 days after infection. Afterwards the titre of the sera and the number of birds reacting fell so that 2 months after infection positive titres were shown by the sera of twelve of the turkeys by the TA test, four by the SA test and only two by the HI test. Three months after infection all the sera were negative by all tests. By the WB test all the turkeys gave positive reactions 6 days after inoculation. After the 12th day this number fell and none of the birds were positive by this test after the 20th day. The sera were anticomplementary up to a dilution of 1/16, and with dilutions greater than this there were no positive ICF reactions. By the MI test the highest titre, 1/4, was obtained between the 6th and 10th day after inoculation but no

birds gave positive sera after the 12th day and two did not react at all. No positive precipitation reactions were obtained at any time during the 3 months after inoculation.

Experiment 5

To study the serological response to re-infection with *M. gallisepticum*, ten of the twenty-six chickens from Expt. 3 were housed separately in two groups of five, 5 months after the initial infection. The birds of one group were re-inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* in C.I.M. containing

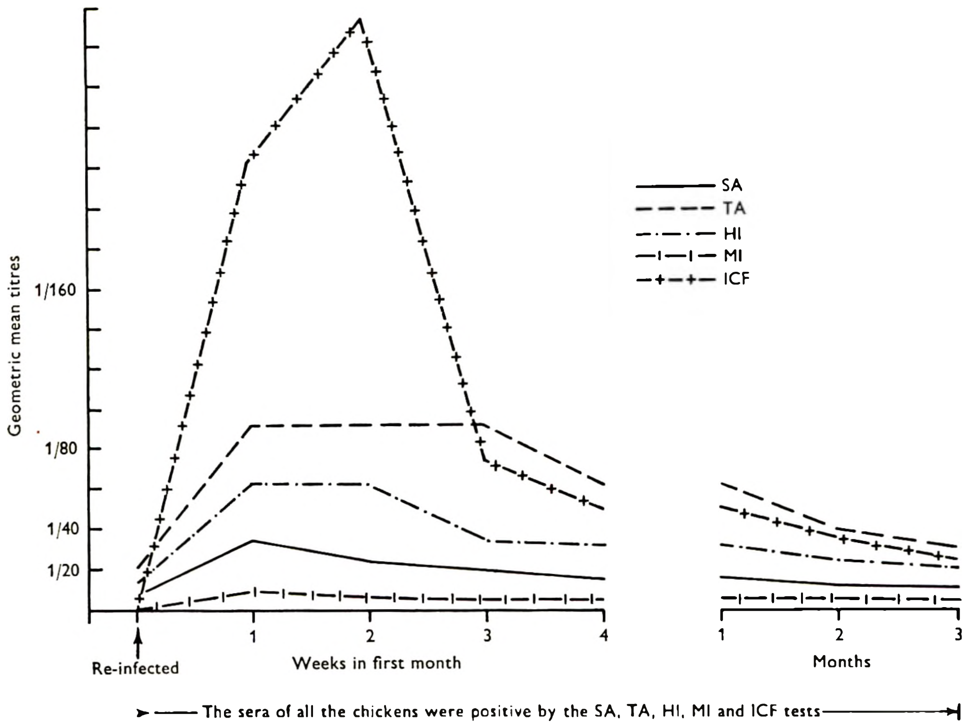

Fig. 6. Geometric mean titres of five chickens re-inoculated intratracheally with a culture of *M. gallisepticum* 5 months after the initial inoculation.

10^{10} viable organisms per ml. At the same time two chickens of the same age but not previously infected with *M. gallisepticum* were similarly inoculated intratracheally and housed with this group. The five birds of the other group were not re-inoculated. All twelve chickens were bled at the time of inoculation, at weekly intervals for 1 month, and then monthly for a further 2 months, and the sera examined.

At the time of re-inoculation the sera of the ten birds from the previously infected group gave positive reactions by the SA, TA and HI tests but antibodies were not detected by ICF, MI or precipitation tests.

The pattern of serological response after re-inoculation is shown in Fig. 6 and followed that obtained after the initial inoculation (Fig. 4). However, titres were higher by one or two dilutions by all tests and persisted at a higher level. Titres by

the ICF test rose and fell rapidly and although the highest titres were obtained with this test, they had fallen by the 4th week below those of the TA test. Titres by the MI test were higher for three of the five birds but the highest titre was only 1/16. By the precipitation test, two lines were obtained with the sera from the five birds at 1 week but not after this time. Positive reactions were obtained for all birds by all except the precipitation test at 3 months, when the experiment ended.

The sera from the five birds which were not re-inoculated showed a gradual fall in titre by the SA, TA and HI tests and no reaction at all by the ICF, MI and precipitation tests. The sera of the other two chickens not previously infected were negative to all tests at the time of inoculation but afterwards gave a positive reaction to all except the precipitation test, the pattern of response being similar to that shown in Fig. 4.

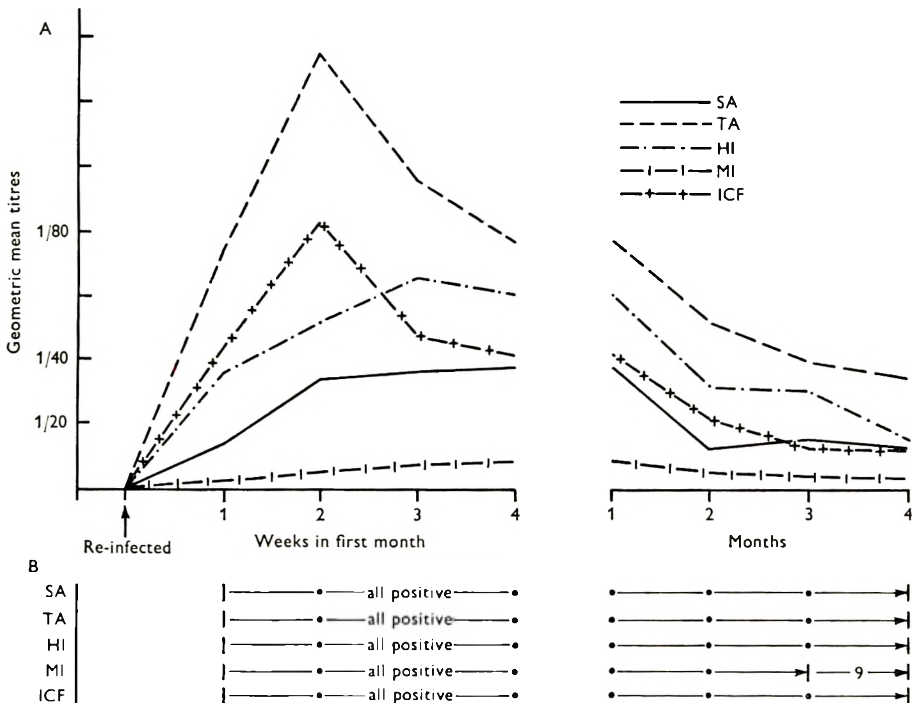

Fig. 7. A. Geometric mean titres of ten turkeys re-inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors by SA, TA, HI, MI and ICF tests.

Experiment 6

To study the serological response to re-infection in turkeys, fifteen of the twenty-one birds from Expt. 4 were housed separately in groups of ten and five respectively, 3 months after the initial infection. Each bird of the group of ten was re-inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* in T.I.M. containing 10^{10} viable organisms per ml. and the remaining group uninoculated. All fifteen turkeys were bled at the time of inoculation, at weekly intervals for 4 weeks, and then at monthly intervals for a further 3 months, and the sera examined.

At the time of re-infection the sera of all birds were negative by all tests for antibodies to *M. gallisepticum* and the pattern of serological response of the ten re-infected turkeys (Fig. 7) followed that obtained after a single infection (Fig. 5). However, after re-infection, the titres obtained by agglutination and HI tests were higher and, despite the anticomplementary nature of the sera, positive reactions were obtained by the ICF test. With this test a rapid rise and fall in titre was seen but, unlike the pattern with chicken sera (Figs. 4 and 6), peak titres were not as high as with the TA test. Although titres by the MI test were higher than after a single inoculation they never exceeded 1/16 and this was observed in only five of the ten birds. By the precipitation test one line was obtained with the sera of each of the ten re-inoculated turkeys 1 week after re-infection and three of the birds gave a positive precipitation reaction for as long as 3 weeks. All the birds gave positive reactions except by the MI and P tests up to 4 months, when the experiment ended. The five turkeys which were not re-inoculated remained negative to all tests.

Experiment 7

The serological response of chickens to immunization with *M. gallisepticum* was studied and compared with the response in rabbits.

An emulsion of *M. gallisepticum* grown in C.I.M. was prepared with Freund's complete adjuvant by mixing equal volumes of a culture of the organisms washed in phosphate-buffered saline pH 7·2 and concentrated 10 times, and adjuvant. Two ml. of the emulsion was inoculated subcutaneously into each of ten 18-month-old Brown Leghorn cockerels; 3 weeks later, each was given at weekly intervals six intravenous 1 ml. injections of the washed concentrated culture, which had been stored at $-27°$ C. The first two injections were the concentrated suspension as used previously, while the 3rd and 4th were twice as concentrated and the 5th and 6th were four and five times as concentrated respectively.

The birds were bled before inoculation, 1 week after the 6th intravenous inoculation and every 4 weeks for 2 months. Because at this stage the titre of the sera by the MI test was very low compared with that obtained for rabbit sera, one additional intravenous inoculation consisting of twice as many organisms as in the 6th intravenous inoculation was given to each chicken. The birds were bled 1 week later and then every 4 weeks for 5 months.

Three rabbits were immunized with the same organism and by the same methods as used for chickens except that the organism was grown in R.I.M. and no additional intravenous injections were given after the 6th. They were bled at the time of the initial inoculation and 1 week after the last intravenous injection and the sera examined.

The general pattern of response in chickens was similar to that following one or two intratracheal inoculations (Figs. 4 and 6). The pre-inoculation sera of the chickens were negative but 1 week after the 6th intravenous injection peak titres were observed (Fig. 8). Titres subsequently fell, but 1 week after the re-inoculation at the 9th week they peaked even higher than before and then fell again. The highest titres were obtained with the ICF test but they fell rapidly and were little higher than by the TA test at 9 weeks after the first series of inoculations. Following

re-inoculation at the 9th week titres by the ICF test again peaked higher than by the other test and did not again return to the level of the TA titres for 17 weeks. By all tests, titres were generally two or three dilutions higher than the corresponding ones following two intratracheal inoculations. Although titres were higher by the MI test than following intratracheal inoculations, they were rarely

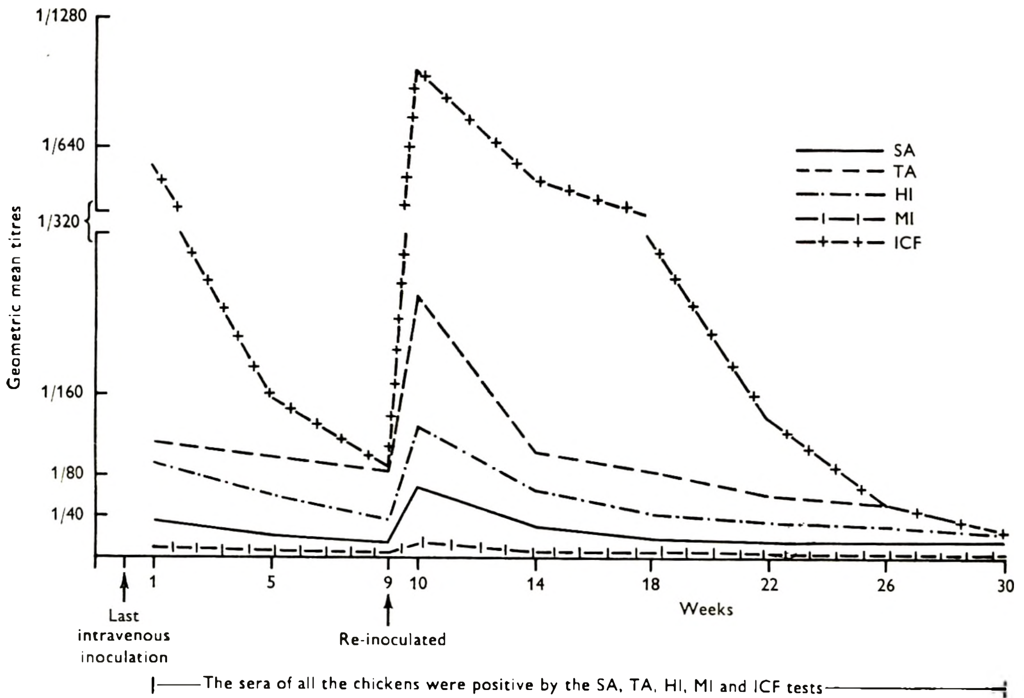

Fig. 8. Geometric mean titres of ten chickens hyperimmunized with a culture of *M. gallisepticum*.

Table 2. *Geometric mean peak titres of ten chickens, and geometric mean titres of three rabbits immunized with A* 514 *strain of* M. gallisepticum

	Test				
	SA	TA	HI	ICF	MI
Chicken	1/70	1/260	1/130	1/1024	1/14
Rabbit	1/127	1/640	1/403	Not done	1/812

higher than 1/32 and then only for the serum of one bird. By the precipitation test the sera were initially positive with three or four lines, but these gradually diminished in number and after 9 weeks the sera from two of the birds gave no precipitation at all. Following re-inoculation at the 9th week, all birds again showed between three and five lines of precipitation; and, although the number of lines declined, seven of the ten birds were still positive 21 weeks later.

The rabbit sera examined at 1 week after the 6th intravenous injection by the SA, TA, and HI tests showed titres higher by one or two dilutions than the chicken

sera even after the re-inoculation of the chickens at the 9th week (Table 2). In contrast to the low titres obtained with the chicken sera by the MI test the titres with rabbit sera were five or six dilutions higher. By the precipitation test three lines were seen with each serum.

DISCUSSION

Using the methods described in this paper for preparing and standardizing reactants and for performing the tests and using experimental birds housed in isolation and free from respiratory viruses and *M. gallisepticum*, all the pre-inoculation sera were negative. It was therefore assumed that a reaction by any of the tests, no matter how low the titre, was specific for *M. gallisepticum*.

The pattern of response after a single intratracheal inoculation of chickens showed that the highest titres were obtained with the ICF test, followed by the TA, HI, SA and MI tests. This is in keeping with the results of Boulanger & Rice (1953), who examined the sera of chickens for antibodies to Newcastle disease virus and found that higher titres were obtained by ICF than by the HI test.

With the ICF test a rapid rise was followed in 3 or 4 weeks by a rapid fall to below the titres of the TA test even when the chickens were inoculated on two occasions. However, after multiple inoculations the elevation of the ICF titre above that of the TA titre was maintained for a longer period. The titre of paired sera taken at an interval of about a week might be of value in indicating whether an infection or re-infection is recent. A disadvantage of the test as undertaken by us was that all chicken and turkey sera were found to be anticomplementary up to a dilution of 1/8 and 1/16 respectively, so that unless the amount of complement in the test was increased, thus reducing the peak titres, the test would be of little value in detecting low levels of antibody. It is interesting that in turkeys peak titres following two inoculations were not as high by the ICF test as by the TA test.

The titres obtained by the TA test were higher and persisted longer than those obtained by the HI and SA tests but the general pattern of response to single or double intratracheal inoculations in chicken and turkey sera and to multiple inoculations in chicken sera was similar by all three tests. This is in agreement with the results of Adler (1958), who found higher titres by the TA than by the SA test with turkey sera, and Baharsefat & Adler (1965), who obtained higher titres by TA than by HI with chicken and turkey sera taken soon after infection but the reverse with sera taken later. Hofstad (1957) and Rhoades *et al.* (1965), however, found titres by the HI and TA test to be similar for turkey sera but Hofstad observed positive reactions by the HI test to persist for a longer period. In contrast Jungherr *et al.* (1955) detected a larger number of reactors with chicken sera by the SA than by the HI or TA test.

Adler & DaMassa (1964) and Baharsefat & Adler (1965) found the antiglobulin test more sensitive than the TA or HI test.

It should be pointed out that the amount of haemagglutinating antigen used in the HI test will influence its sensitivity. Jungherr *et al.* (1955) used 2 HA units standardized to 100% end-point and Hofstad (1957) used 2 HA units, but the

end-point is not indicated. Baharsefat & Adler (1965) used the same technique, whereas we used 4 HA units standardized to 100% end-point. We found it essential to use this quantity of antigen in order to obviate non-specific inhibitors, and found it more effective than using receptor-destroying enzyme. Newnham (1964) also used 4 HA units for this purpose but no indication is given of the end-point in the standardization. In the standardization and in the test itself the 50% end-point should give greater accuracy but we found that 100% end-point was easier to determine when reading the test.

Although the SA test was found to be less sensitive than the TA or HI test, it has the advantage of being rapid and easily performed and therefore can be utilized as a routine flock test. The time interval between mixing the reactants and reading the test is influenced not only by the ambient temperature but also perhaps by the nature of the antigen, which in turn will be influenced by such factors as the strain of organism, and the method of culture, harvesting and standardization. Differences in these may account for the differences in the recommended time interval. For instance, Adler (1954) and Jerstad, Hamilton & Smith (1959) suggested that this period should be 5 and 8 min. respectively for turkey sera. These factors may also account for the unsatisfactory results in the test obtained by Jungherr *et al.* (1955) and Hofstad (1957), both with turkey sera.

The whole blood test was the least sensitive of the agglutination tests. Our technique closely followed that of Jungherr *et al.* (1955), using an equal volume of blood and stained antigen. Aftosmis, Tourtellotte & Jacobs (1960) drew attention to the importance of the ambient temperature and in our hands it never fell below 65° F. and we found no increase in the number of reactors by prolonging the time of the test beyond 2 min. for chickens and 3 min. for turkeys, when using the antigen prepared by ourselves or a commercially available one.* Despite the relatively smaller number of reactors and the relatively shorter period of their detection by this test it has several advantages. It is simple and easy to use, it is rapid and can be performed on the farm, and the birds need be handled only once. Dust on the slide may interfere with reading of the test and adequate precautions should be taken in a dusty environment.

With the MI test only very low titres were obtained even after two intratracheal inoculations of chickens and turkeys and after multiple inoculations of chickens. On the other hand sera from rabbits after multiple inoculations showed a high titre for MI antibodies. This would indicate that either only low-titre sera for the MI test can be prepared in chickens or turkeys, using our inoculum, or that a co-factor is necessary in the test. Unheated horse serum is an essential co-factor for the demonstration of fermentation-inhibiting antibodies in rabbit serum to *M. pneumoniae* and to *M. fermentans*, and to the Negroni agent, and unheated guinea-pig serum is an additional essential co-factor for the Negroni agent (Taylor-Robinson, Purcell, Wong & Chanock, 1966) .In our hands the addition of unheated horse and guinea-pig sera did not increase the MI titre of chicken, turkey and rabbit antiserum.

* Burroughs Wellcome.

Precipitation lines were produced by only a small proportion of chickens and none of the turkeys after one intratracheal inoculation whereas they were observed in the sera of both species 1 week after two or more inoculations and for a few weeks afterwards.

In our experiments antibodies could be detected in all chickens and turkeys within 1 week of infection by the TA, SA and HI tests for chicken and turkey sera and by the ICF test for chicken sera. Peak titres were recorded by these tests between the first and second week. This is in contrast with the observations of Fahey & Crawley (1954), who infected day-old chickens by intranasal drop of an infected tissue suspension containing 'a PPLO and the virus previously described', probably *M. gallisepticum* and the Fahey–Crawley virus, and found that positive titres by the HI test first appeared at 9 days for some birds and by 29 days all were positive, and peak titres appeared after 10 weeks. Newnham (1964) infected chickens and turkeys with exudate from stock infected with the A514 strain of *M. gallisepticum* and found that positive titres by the HI test were observed in the chickens after 3 weeks and in turkeys after 1 week and that peak titres were obtained at 6–9 weeks and 3–9 weeks respectively. Roberts (1964*a*, *b*) inoculated groups of chickens with broth culture of the S6 strain of *M. gallisepticum* via the intranasal sinuses, the trachea, and posterior abdominal air sacs, and a group of turkey poults via the infra-orbital sinuses. In both chickens and turkeys maximum titres by the HI test were observed 4 weeks after infection.

The titres for chicken and turkey sera and the duration of positive reactions with turkey sera recorded by us have been less than those obtained by other workers. For instance, Fahey & Crawley (1954) observed peak titres by the HI test of 1/160 in chicken sera, and with turkey sera Hofstad (1957) and Crawley (1960) reported peak HI titres of 1/320 and 1/640 respectively and positive HI titres for 11–15 months. Newnham (1964), also using the HI test, observed peak titres with chicken and turkey sera of 1/640 to 1/5120, and Roberts (1964*a*, *b*) found peak titres by the HI test of 1/160 for both chickens and turkeys and positive reactions in most turkeys for at least 19 months.

The earlier appearance of positive reactions and peak titres in our experiments may be due to the large number of organisms in the initial inoculum. In addition it should be pointed out that the peak titres obtained with both chicken and turkey sera in our experiments were no greater than the titres observed by Newnham (1964) at the corresponding period after infection, but in Newnham's experiments the titres continued to rise for several weeks. The higher and later peak titres and the longer duration of a positive response seen by Fahey & Crawley (1954), Newnham (1964) and Roberts (1964*a*, *b*) are very probably associated with the virulence of the organisms in the inoculum. In our experiments the organism had possibly lost virulence by repeated culture in artificial medium and there was no evidence of disease in the infected chickens or turkeys, whereas Fahey & Crawley (1954) and Newnham (1964) observed symptoms and lesions, and the rise of HI antibody closely followed the development of clinical disease. Adler, Shifrine & Ortmayer (1962) infected turkeys by intranasal and intraperitoneal inoculation and examined the sera by SA, TA, and HI tests and concluded that absence of

lesions was usually reflected in absence of, or only low-titre antibody and that high titres of 1/80 and above would generally indicate disease rather than infection in which symptoms and macroscopic lesions were absent.

Roberts (1964*b*) examined the sera of eighty-six turkeys 19 weeks after infection, and noted that of sixty-three with positive titres by the HI test forty-four showed lesions, but all of twenty-three with no reactions to the test showed no lesions.

It is unlikely that the site of inoculation of our experimental birds was associated with the relatively low titres obtained. Intratracheal inoculation was chosen because there was less probability of wastage of organisms than following inoculation into the infra-orbital sinus or by nasal instillation as was practised by most other workers. Furthermore, Roberts (1964*a*) found that following the inoculation of three groups of chickens in the nasal sinus, trachea and posterior abdominal air sac respectively, the highest titre by the HI test was seen in many more of the birds inoculated via the trachea than by the other routes. Rhoades *et al.* (1965) inoculated three groups of six turkeys each with a culture of *M. gallisepticum* containing 10^7 organisms per ml. One group was inoculated intratracheally with 0·2 ml. per bird, another group with 0·4 ml. intranasally and the third via the right infra-orbital sinus with 1·0 ml. per bird. A fourth group was infected by exposure to these infected birds. Blood samples were taken weekly and examined by TA and HI tests. The initial antibody response was greatest in turkeys infected by the intratracheal and intrasinal routes but after 4 weeks differences were less obvious.

In our experiments higher titres were observed with chicken than with turkey sera and antibodies persisted for a longer time. Both chickens and turkeys were inoculated with approximately the same number of organisms, which means that the chickens received approximately 4–6 times as large a dose on a live weight basis. If the organisms multiplied in the infected host, however, this should be of little significance and it appears that, with our inoculum, turkeys did not respond as well as chickens. Newnham (1964) found no appreciable difference in the titre of the sera of chickens and turkeys by the HI test after infection with the A514 strain of organism.

Re-infection of chickens by intratracheal inoculation 5 months after the initial infection, when antibodies could only be detected by the SA, TA and HI tests, and similar re-infection of turkeys 3 months after initial infection, when no antibodies could be detected, gave rise to positive reactions by all tests with titres higher than observed after the initial infection. Fahey & Crawley (1954) found that re-infection of chickens by the intranasal route, 77 days after initial infection, increased the titre of pooled sera examined by the HI test from 1/40 to 1/80. Adler *et al.* (1962) infected turkeys by the intranasal route and challenged them by intraperitoneal infection 7 weeks later, when titres were 1/10 or less and there was no clinical sign of disease. Little change in titre was observed when the sera were tested by SA, TA and HI tests. Roberts (1964*b*) re-infected a group of turkeys by inoculation into the infra-orbital sinus 19 weeks after the initial infection, when they showed no clinical signs of disease. He found that there was an antibody response by the HI

test in those turkeys which were negative at the time of re-infection but not in those showing positive titres (1/5 to 1/20) at that time.

It is difficult to explain the apparent lack of response found by these workers, especially in turkeys, to reinfection with *M. gallisepticum* when a positive titre already exists.

The MI test using sera prepared in rabbits may be a useful method for the classification of avian mycoplasma because the test can be performed without having to prepare and standardize antigen. However, it would be necessary to examine the specificity of the test.

Antisera to *M. gallisepticum* can be prepared in chickens which is comparable in titre to that produced in rabbits except for the MI titre.

SUMMARY

A comparison was undertaken of several serological tests in determining the response of chickens and turkeys experimentally infected with the A514 strain of *Mycoplasma gallisepticum.*

After a single intratracheal inoculation of chickens with a culture of the organism, the highest titres were obtained by the indirect complement fixation (ICF) test, followed by the tube agglutination (TA), haemagglutination inhibition (HI), slide agglutination (SA) and metabolic inhibition (MI) tests. By all these tests positive titres were observed within the first week and peak titres between the first and second weeks. At 5 months there was no positive reaction by the ICF test but most chickens gave positive readings by the TA, HI and SA tests for at least 14 months after infection, but turkey sera became negative by all tests after 3 months.

A disadvantage of the ICF test was that sera up to a dilution of 1/8 and 1/16 for chicken and turkey respectively were anticomplementary, and in turkeys this masked the ICF titre, which presumably was low following one intratracheal inoculation. Titres in turkeys with the TA, HI and SA tests followed the pattern seen with chickens and were generally lower than those found by other workers probably because of the avirulent nature of the inoculum used.

The WB test was the least sensitive of the agglutination tests but is useful as a flock test which can be undertaken on the farm.

The MI test gave the lowest titres of all and antibodies could be detected for only 4 months following one intratracheal inoculation. Even with serum prepared by multiple inoculations in chickens the titre was never higher than 1/32 compared with 1/1024 for serum similarly prepared in rabbits.

Precipitins were detected by the agar gel method in the sera of chickens and turkeys after two intratracheal inoculations but in only some of the chickens and none of the turkeys after one inoculation.

By all tests higher titres were observed with chicken than turkey sera and antibodies persisted for a longer time.

Re-infection of chickens when antibodies to the initial infection had become low, and of turkeys when antibodies were no longer detectable, gave rise to an anamnestic response with titres which were higher than before.

Antiserum to *M. gallisepticum* prepared in chickens is comparable with that prepared in rabbits except for low titres by the MI test.

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Studies on the inhibitory properties of sodium thioglycollate on the germination of wet spores of clostridia

BY D. A. A. MOSSEL
Central Institute for Nutrition and Food Research TNO, Zeist, The Netherlands

AND H. BEERENS
Laboratory of Anaerobic Bacteriology, Pasteur Institute, Lille, France

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The use of thioglycollate broth for the testing of sterility of surgical apparatus remains the current medical laboratory practice (Meeks, Pembleton & Hench, 1967), despite repeated warnings of bacteriologists (Hirsch & Grinsted, 1954; Galesloot, 1961) that sodium thioglycollate may inhibit the germination of the spores of certain clostridia. In order to avoid obtaining dangerous, false negative results in the future due to this phenomenon, we report the results of an investigation of this subject.

Spores contained in broth solutions were used as these could be expected not to be more sensitive to toxicity of a culture medium than dry spores occurring on contaminated instruments, catgut, gauze, gloves, etc.

MATERIALS AND METHODS

The clostridia studied were from the culture collection of the Laboratory of Anaerobic Bacteriology, Pasteur Institute, Lille, France. Three groups of organisms were chosen: predominantly proteolytic types (*Cl. bifermentans*, *Cl. histolyticum* and *Cl. sporogenes*), mixed glycolytic-proteolytic strains (*Cl. perfringens* and *Cl. septicum*) and predominantly glycolytic types (*Cl. acetobutylicum*, *Cl. butyricum* and *Cl. tertium*).

Spore-bearing cultures of these organisms were obtained in the following way. The strains were cultivated in an anaerobic jar, using a broth containing meat extract, yeast extract, glucose and meat particles (Mossel *et al.* 1965), until fully grown cultures had been obtained. The tubes were then evacuated, sealed and left at room temperature for about 2 months. These cultures were then heated for 10 min. at 80° C. before use in our experiments. Such a heat treatment will certainly kill all vegetative cells; slight losses of spores may also occur (Roberts, Gilbert & Ingram, 1966), but these are not important in these experiments.

The following four media were used for the spore counts, for comparison with the reference medium.

Medium 1. Thioglycollate agar U.S.P. (Pittman, 1946; *U.S. Pharmacopoeia*, 1965). Tryptone, 15 g.; yeast extract, 5 g.; glucose, 5·5 g.; sodium thioglycollate,

A.R., 0·5 g.; sodium chloride, 2·5 g.; cystine hydrochloride, 0·5 g.; agar, 15 g.; glass-distilled water, 1 l.; pH = $7{\cdot}2 \pm 0{\cdot}1$.

Medium 2. As medium 1, but without thioglycollate; indicated in Table 1 as U.S.P. – TH.

Medium 3. Thioglycollate agar, Difco (*Difco Manual*, 1953). Casitone, 15 g.; yeast extract, 5 g.; sodium thioglycollate, A.R., 0·3 g.; cystine hydrochloride, 0·25 g.; agar, 15 g.; glass-distilled water, 1 l.; pH = $7{\cdot}2 \pm 0{\cdot}1$.

Medium 4. As medium 3, with glucose 5 g./l. added.

As the reference medium agars containing cysteine (Quastel & Stephenson, 1926) were used. These, in all instances, contained 0·5 g./l. cysteine-HCl, 5 g./l. yeast extract and 2·5 g./l. disodium phosphate. For the proteolytic clostridia 2·5 g./l. tryptone and 3 g./l. meat extract paste were used as further sources of nitrogen, whereas for the glycolytic types 15 g./l. soya peptone was preferred instead; these had been established to be the optimal media in the respective instances in an earlier investigation (Mossel *et al.* 1965).

Counts were made in oval cross-section tubes in triplicate. Only those dilutions were taken into account that contained between 30 and 200 colonies. Incubation was for up to 3 days at 37° C. in all instances.

RESULTS AND DISCUSSION

The results obtained are shown in Table 1. These data have been evaluated there by the introduction of the ratio R = count in reference cysteine agar, divided by the count in a given other medium.

Comparing U.S.P.-thioglycollate agar (medium 1) with the reference medium shows that it is inadequate for the spores of eight strains of clostridia, including all the proteolytic types, poor for three strains, suboptimal for two strains and satisfactory for one. Difco thioglycollate agar (medium 3) was inadequate for the spores of five strains, suboptimal for another five, but acceptable for four others. Hence the Difco formula appeared to be considerably better than the U.S.P. This may be due to the richer type of peptone used in the former medium, because omitting the thioglycollate from the U.S.P. medium resulted in a decisive improvement in only four cases, leaving the counts of the spores of most proteolytic clostridia at a level 10^{-3}–10^{-4} times the reference counts.

It is a striking fact that the addition of glucose to the Difco formula led to a considerable improvement in count with four strains. In fact, the Difco formula plus glucose was only quite inadequate for *Cl. histolyticum* and definitely suboptimal for one strain of *Cl. acetobutylicum*; the recoveries of the other clostridia were all virtually within the region $R < 3$. It seems, finally, worth while to stress that only in two intances was R less than 1·0. Both the two occasions were with strain *Cl. butyricum* 1375 and R remained over 0·5, which is still a quite acceptable recovery.

The results of these experiments confirm and extend earlier observations of the great superiority of media containing cysteine (Hirsch & Grinsted, 1954; Galesloot, 1961). The conclusions for medical laboratory practice must be that: (i) the use of sodium thioglycollate in sterility test media should be discontinued; (ii) it should be replaced by cysteine hydrochloride.

Table 1. *Comparative spore counts of fourteen strains of various metabolic types of* Clostridium, *made in media with and without thioglycollate*

(The figures in parentheses indicate the ratio R = [count in reference medium]/[count in TH type medium]).

Strain		Spore count per ml. × 100: Thioglycollate type media: USP	USP – TH	Difco	Difco + gluc.	Reference cysteine agar
Cl. acetobutylicum	A 1	< 0·01 (+ +)*	< 0·01 (+ +)	0·05 (+ +)	27·6 (2·4)	67·4
	A 23	0·09 (51)	2·0 (2·3)	< 0·01 (+ +)	0·5 (9·2)	4·6
	A 462	< 0·01 (+ +)	< 0·01 (+ +)	0·2 (+ +)	22·6 (1·2)	27·5
Cl. butyricum	T	5·6 (1·1)	1·9 (3·4)	2·6 (2·5)	2·6 (2·5)	6·4
	1375	1·6 (7·8)	18 (0·7)	8·8 (1·4)	14·2 (0·9)	12·4
Cl. tertium	Ut	0·3 (33)	4·2 (2·3)	1·1 (9)	8·5 (1·2)	9·9
Cl. perfringens	8238	1·7 (2·6)	2·4 (1·9)	3·7 (1·2)	3·2 (1·4)	4·5
	166/63	0·6 (13)	4·9 (1·6)	3·9 (2)	7·2 (1·1)	7·9
Cl. septicum	SE 05	< 0·01 (+ +)	< 0·01 (+ +)	1·6 (1·9)	2·1 (1·4)	3·0
Cl. bifermentans	701	< 0·01 (+ +)	< 0·01 (+ +)	4·7 (2·2)	3·0 (3·4)	10·3
Cl. histolyticum	Co	< 0·01 (+ +)	< 0·01 (+ +)	< 0·01 (+ +)	< 0·01 (+ +)	28·0
Cl. sporogenes	1039	< 0·01 (+ +)	< 0·01 (+ +)	3·6 (7·5)	22·4 (1·2)	27·2
	13499	< 0·01 (+ +)	< 0·01 (+ +)	< 0·01 (+ +)	21·2 (2·4)	51·8
	72840	< 0·01 (+ +)	0·8 (14)	3·2 (3·5)	8·2 (1·4)	11·2
No. of strains for which R is:						
satisfactory, i.e. ≤ 2		1	3	4	8	—
suboptimal, i.e. 2·1–10		2	3	5	5	—
poor, i.e. 10·1–100		3	1	0	0	—
inadequate, i.e. > 100		8	7	5	1	—

* (+ +) indicates > 100.

SUMMARY

Wet spores of fourteen strains of *Clostridium*, representing eight species, were enumerated in four different types of thioglycollate agar and in a similar medium in which thioglycollate was replaced by cysteine-HCl. Thioglycollate appeared to be toxic, in principle, to almost all strains tested, although the degree of toxicity was influenced by other components of the medium. These experiments entirely confirm earlier observations and must lead to the conclusion that the use of thioglycollate in sterility test media should be discontinued and that cysteine-HCl should be used as the redox potential reducing compound instead.

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Evidence for a two-stage model of microbial infection

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

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Any model for microbial infection must account for the heterogeneity in response that invariably characterizes infections of all kinds. Some hosts die, and at different times, while others survive: and at every stage of infection the number of viable organisms differs from individual to individual, even under experimental conditions where dosage and factors like the age and weight of the hosts are kept constant. This heterogeneity can be considered as arising in two ways: either it could be fully established almost immediately after inoculation (e.g. if the inoculated organisms were randomly distributed amongst susceptible and resistant sites in the host: Meynell & Stocker, 1957; Meynell, 1957), or it could increase progressively as time passed (e.g. if infection followed a birth–death process: Williams, 1965 *a*, *b*; Armitage, Meynell & Williams, 1965; Williams & Meynell, 1967; Shortley & Wilkins, 1965). The first possibility was suggested by viable counts on mice infected with *Salmonella typhimurium* which showed that the counts made only 1·5 hr. after inoculation already differed considerably and that the degree of heterogeneity did not appear to increase during the following 10–14 days. A large number of other colony counts was then examined, with similar results. Thus, it appears that salmonella infections comprise two stages: (1) an initial stage lasting no more than a few hours in which heterogeneity is established; and (2) the subsequent stage in which the degree of heterogeneity often remains unchanged so that the infection may be progressing in much the same way in all the hosts. It will be seen that this interpretation corresponds to the course of events suggested by Miles (1963) on quite different grounds: namely, that there is an early 'decisive period' in which primary lodgement of part of the inoculum occurs, followed by multiplication of the surviving organisms.

Figure 1 shows the two extreme cases to be discussed: in Fig. 1 *a*, the colony counts (n) differ more and more as time passes, whereas in the two-stage model of Fig. 1 *b*, the scatter is constant once the initial period is past. In each case, it is assumed that log n follows a parabola; that an individual falls ill or dies if n reaches the morbidity or mortality threshold respectively (Williams & Meynell, 1967); and that the mean rate of increase in log n at a given time is the same for all doses (Williams & Meynell, 1967; Mackaness, 1962, fig. 2). In fatal infections, n will appear to increase exponentially, as suggested by the linear relation generally observed between log dose and mean response time (see Meynell & Meynell, 1958; Shortley & Wilkins, 1965).

The experiments from which the colony counts were derived are shown in

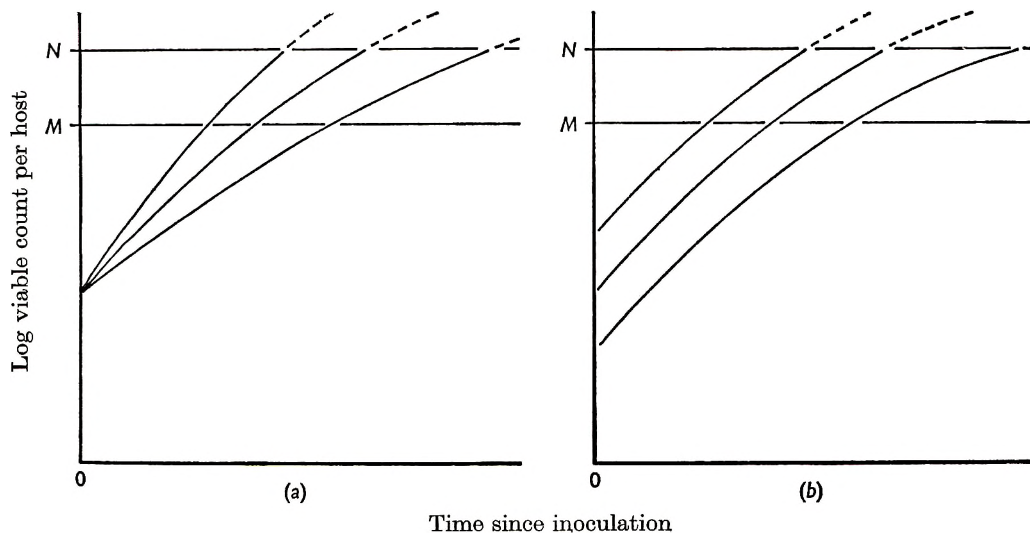

Fig. 1. Two models for the origin of heterogeneity in microbial infection. Each assumes that individual viable counts follow a parabolic course and that the host falls ill or dies if the count reaches the morbidity threshold (M) or the mortality threshold (N). In (a) individual counts differ more and more as time passes; whereas, in (b), these differences are fully established within a short time of inoculation.

Table 1. *Details of the experimental infections*

	Dose in number of		No. of counts based on x colonies								
Organisms	Organisms	LD 50	1–10	11–20	21–30	31–40	41–50	51–100	101–300	> 300	Reference
S. typhi	10^7	10^{-1}	—	—	1	4	5	10	12	12	Meynell (unpubl.)
S. typhimurium	2×10^6	$6{\cdot}3\times10^3$	—	—	—	—	—	—	2	10	Meynell & Meynell (1958), Fig. 10a
	8×10^4	$1{\cdot}3\times10^2$	—	—	1	—	—	1	3	13	Meynell & Meynell (1958), Fig. 10b
	3×10^3	$2{\cdot}6\times10^{-1}$	—	1	2	3	—	3	15	15	Meynell & Meynell (1958), Fig. 10c
S. paratyphi B	5×10^5	5×10^{-2}	3	—	—	6	3	17	17	6	Meynell & Meynell (1958), Fig. 11
	3×10^5	$1{\cdot}2\times10^{-4}$	—	2	1	3	1	17	23	12	Williams & Meynell (1967)
	3×10^5	$1{\cdot}2\times10^{-4}$	1	—	—	1	2	22	26	7	Williams & Meynell (1967)
	7×10^2	$4{\cdot}8\times10^{-2}$	5	3	3	1	3	8	19	17	Williams & Meynell (1967)
	9×10^2	$4{\cdot}8\times10^{-2}$	6	6	4	1	5	10	12	12	Williams & Meynell (1967)
S. typhimurium	10^7	5×10^{-1}	—	—	—	—	—	2	6	6	Maw & Meynell (1968), Exp. 1
	10^7	5×10^{-1}	1	—	—	1	—	6	17	6	Maw & Meynell (1968), Exp. 2
	10^7	5×10^{-1}	—	—	4	1	—	12	30	24	Maw & Meynell (1968), Exp. 3
	10^7	5×10^{-1}	—	—	—	—	1	8	17	12	Maw & Meynell (1968), Exp. 4

Table 1. The counts were analysed by calculating $\sigma_{\log n}$, the standard deviation of log n, for each time after inoculation. Values of log n rather than n were used because plots of the individual counts show that log n is clearly more symmetrically distributed than n itself (e.g. Hobson, 1957; Meynell & Meynell, 1958; Williams & Meynell, 1967). The values of $\sigma_{\log n}$ were then plotted against time since inoculation (Fig. 2) and showed that their values fluctuated around a mean of 0·8–1·0

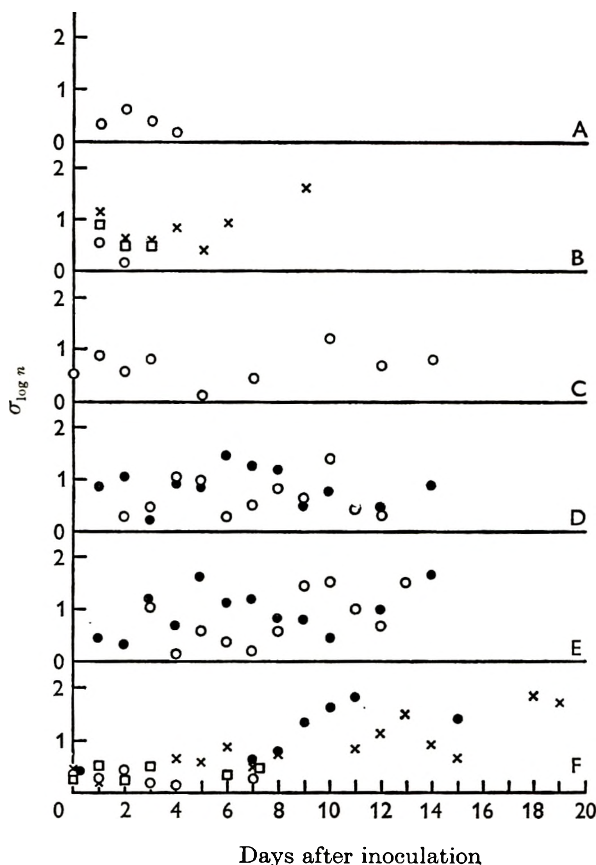

Fig. 2. Values of $\sigma_{\log n}$ observed in the experiments of Table 1. A: *Salmonella typhi* (Meynell, unpublished). B. *Salm. typhimurium* (Meynell & Meynell, 1958. ○, Fig. 10*a*; □, Fig. 10*b*; ×, Fig. 10*c*). C: *Salm. paratyphi* B (Meynell & Meynell, 1958, Fig. 11). D,E: *Salm. paratyphi* B (Williams & Meynell, 1967; Figs. 2*a*,*b*, respectively. The points come from duplicate experiments, indicated by ○, ●). F: *Salm. typhimurium* (Maw & Meynell, 1968: ○, Exp. 1; □, Exp. 2; ×, Exp. 3; ●, Exp. 4.).

during the first 10 days, although they might increase subsequently. The observed scatter in n arose almost entirely from the nature of the infection and not from trivial causes like the sampling error of the doses or colony counts, since the numbers of organisms per dose and the numbers of colonies counted were so large that sampling error was negligible (Table 1). For example, a typical value of $\sigma_{\log n}$ was derived from counts on five mice. Supposing each count to be based on x colonies, then $\sigma_{\log n} \simeq 0{\cdot}4343/\sqrt{5x}$. Hence, the values of $\sigma_{\log n}$ for groups of five

counts, each based on 10, 20, 40 or 100 colonies, are 0·061, 0·043, 0·031 and 0·019 respectively, which are considerably less than the observed values of approx. 0·8 shown in Fig. 2. The constancy of $\sigma_{\log n}$ for the first 10 days therefore suggests that during this period at least, these salmonella infections are better described by the two-stage model of Fig. 1*b*, in which $\sigma_{\log n}$ is constant, than by the alternative model of Fig. 1*a*, in which $\sigma_{\log n}$ increases progressively from zero. After 10 days, $\sigma_{\log n}$ sometimes increased, at about the time the viable counts began to decline, possibly because new host processes then appeared which increased the heterogeneity in count by having a different intensity or time of onset in different individuals.

The validity of the two-stage model can, in principle, be tested further, by determining how the percentage of responses varies with size of dose and by examining the times at which individual hosts respond to inoculation of a given dose.

The dose-response curve

In general, the larger the number of organisms inoculated, the larger the proportion of hosts that subsequently respond. Both models imply that this occurs because, as the dose is increased, a growing proportion of counts reach the morbidity or mortality thresholds. On the two-stage model, the distribution of n from the end of the decisive period determines the distribution of response on dose. In practice, log n appears to be symmetrically distributed, so that response will be symmetrically distributed on log dose, as is observed (see Meynell, 1957). If log n differs considerably from host to host, a given increment in dose will produce a smaller increase in the proportion of hosts responding than would be the case if log n differed less. The standard deviation of log n (namely, $\sigma_{\log n}$) therefore determines the slope of the log dose-response curve which in turn determines the values of $\sigma_{\log \mathrm{ED}50}$, the standard deviation of the log dose causing 50 % of hosts to respond. In fact, since the response thresholds are constant and the viable counts appear to increase exponentially in fatal infections, $\sigma_{\log n} = \sigma_{\log \mathrm{ED}50}$. Values of $\sigma_{\log \mathrm{ED}50}$ have been determined for many infections and are always 0·5 or greater (Meynell, 1957) which is consistent with the values of $\sigma_{\log n}$ shown in Fig. 2. In other words, frequency of response increases with increase in dose to about the extent predicted from the scatter in counts.

Distributions of individual response time (t)

It follows from Fig. 1*b* that a symmetrical distribution of log n leads to a symmetrical distribution of t. At first sight, this is inconsistent with the distributions found in practice, many of which can be reasonably well depicted as normal distributions of log t (see Meynell, 1963; Sartwell, 1950, 1966). The explanation comes from Sartwell's investigations of the distributions observed in naturally occurring outbreaks, which showed that although t differed, it did so to only a limited extent. Sartwell assumed a normal distribution of log t, and could therefore express its spread by Δ, the 'dispersion factor':

$$\Delta = t_{50}/t_{16} = t_{84}/t_{50},$$

Table 2. *Values of Δ observed in experimental infections*

Pathogen	Host	Δ	Author
Mouse encephelomyelitis virus	Mice	1·3	Gard (1940)
Poliomyelitis virus	Mice	1·5	Gard (1943)
Streptococcus pneumoniae	Mice	1·28	Cavalli & Magni (1943)
Mycobacterium tuberculosis	Mice	1·1	Martin (1946)
M. tuberculosis	Mice	1·2	Litchfield (1949)
Leukosis virus	Chickens	1·42	Eckert, Beard & Beard (1951)
Avian erythromyeloblastic leukosis virus	Chickens	1·11	Eckert, Beard & Beard (1954)
Salmonella dublin	Mice	1·53	Reid & Macleod (1954)
Avian erythroblastosis virus	Chickens	1·15	Eckert, Beard & Beard (1956)
Salm. gallinarum	Chickens		
	(Barred Rock)	1·4	Williams Smith (1956)
	(Light Sussex 3)	1·33	Williams Smith (1956)
Pasteurella pestis	Guinea-pig	1·37	Druett *et al.* (1956)
Bacillus anthracis	Mice	1·2	Roth, DeArmon & Lively (1956)
Salm. typhimurium	Mice	1·38	Meynell & Meynell (1958)
Salm. typhimurium	Mice	2·00	Meynell & Meynell (1958)
Salm. typhimurium	Mice	1·33	Meynell & Meynell (1958)

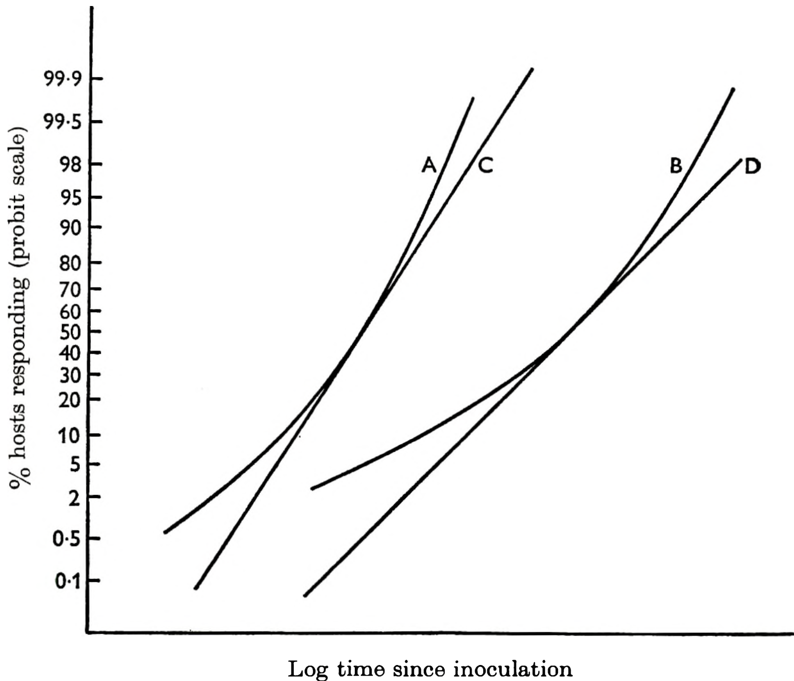

Fig. 3. A comparison of normal distributions of t and of log t. Curves A, B: normal distributions of t with $\sigma_t = 1{\cdot}0$ and $1{\cdot}7$, respectively. Curves C, D: normal distributions of log t with $\Delta = 1{\cdot}3$ and $1{\cdot}5$, respectively. Curves A and C (or B and D) would be indistinguishable in practice unless an exceptionally large number of hosts was used.

where t_{16}, t_{50}, and t_{84} are the times corresponding to 16, 50 and 84 % responses. Thus, $\Delta = 1$ if all the responses occur simultaneously and its value rarely exceeds 1·5 in outbreaks. The scatter of t is also limited in experimental infections (Table 2). Because of this, the corresponding normal distributions of t, log t and $1/t$ will be extremely hard to distinguish in practice because their differences are most marked at the tails of the distributions which can rarely be determined precisely because of the large number of hosts required. Thus, normal distributions of log t with $\Delta = 1{\cdot}1$ and 1·5 give virtually linear curves when $1/t$ is plotted (Meynell & Williams, 1967) and, as Fig. 3 shows, analogous normal distributions of t appear almost linear when plotted on a logarithmic time scale. The empirical use of log-normal distributions of t is therefore unlikely to be inconsistent with t being symmetrically distributed, as suggested by Fig. 1*b*. Thus, the dose-response curves and distributions of individual response times generally observed in bacterial infections are not inconsistent with the distributions of viable counts found in these experiments.

A two-stage process therefore provides as good a description of salmonella infections as any previous model. It could, of course, be tested far more rigorously by obtaining many more values of n and t and by determining the dose-response curve in the same experiment, although, to be useful, this might have to be done on a very large scale. Considered purely as a model, a two-stage process has the disadvantage of a large number of parameters. In addition to the morbidity and mortality thresholds, there are $\sigma_{\log n}$ and the mean proportion of the dose that survives the decisive period, for the first stage, and, for the second stage, the true division and death-rates of the organisms *in vivo* and the rates at which these change with the passage of time.

The existence of two stages is not implausible. The inocula were given by intraperitoneal or intravenous injection, and the bacteria were therefore initially in the peritoneal cavity or the blood, while subsequently they were confined to the organs. The presence of two stages is also indicated by measurements of the true rate of division of *Salmonella typhimurium* in the mouse spleen, where the true mean generation time is 90 min. during the 1·5 hr. after inoculation but thereafter increases to 5 hr. or more (Maw & Meynell, 1968). Whatever the antimicrobial mechanisms of the host may be, the first stage appears 'decisive' in the sense suggested by Miles (1963). During this phase, a varying proportion of the inoculum is killed within a short time, leaving a fraction of survivors whose numbers in part determine the mortality. The fact that two unrelated investigations, one concerned with organisms injected intravenously or intraperitonally and the other with infections of the skin (see Miles, 1956, 1963), have each led to a two-stage model is a strong indication that analogous processes may well come to be found of importance in determining the outcome of other types of infection.

SUMMARY

Colony counts on mice given the same number of *Salmonella* always differ considerably. However, the standard error of the mean log count does not increase

after the first 1·5 hr. of infection until the 8th or 10th day. These infections therefore appear to pass through an initial stage lasting a few hours, in which a varying proportion of the inoculum is killed, followed by a prolonged second stage in which the scatter in individual colony counts remains constant.

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Sources of salmonellas in market swine*

BY L. P. WILLIAMS, JR. AND K. W. NEWELL†
Division of Epidemiology, Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans, Louisiana

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INTRODUCTION

Pigs at slaughter and pig products are frequently infected with *Salmonella* and are therefore a possible source of salmonella infection in man (Felsenfeld, Young & Yoshimura, 1950; Galton, Lowery & Hardy, 1954; Wilson *et al.* 1961; Williams, 1965). If this contamination of human food is to be controlled the sources of pig infections need to be ascertained and described, as terminal treatment of these products prior to retail sale is impracticable at this time.

Several investigators have reported an increase in the proportions of pigs found to be excreting salmonellas at the farm and in the holding pens before slaughter, and in the infection rate after slaughter. Galton *et al.* (1954) recovered salmonellas from 7·2, 15 and 51 % of pigs sampled in these three locations. American Meat Institute researchers reported 2·7 % of pigs on the farm, 94 % of faecal specimens in the abattoir pens, and 43 % of pigs at slaughter to have salmonellas (Leistner *et al.* 1961). McDonagh & Smith (1958) reported 2·9 % of pigs tested excreting salmonellas in holding pens, and 13 % infected after slaughter. These investigations were done in different areas, by different investigators using different methods. They were of cross-sectional design, comparing unlike pig populations, often at different times and places.

While it seems probable that most farm infections are the result of salmonella contamination of feed, and that new infections in the marketing process and in the holding pens are due to indirect spread (from pig via the environment to pig) or possibly direct spread from pig to pig, this has not been clearly demonstrated. At the time our investigations were begun we were aware of only one longitudinal study in the literature. Shotts, Martin & Galton (1962) demonstrated a build-up of swine infections by comparing salmonella excretion of cull sows at a sale barn, in abattoir pens after transport, and after slaughter as demonstrated by rectal swabs. They did not state whether the trucks contained salmonellas that could account for this build-up. Further, they did not report the serotypes recovered from the holding environment or the pigs after slaughter in such a way that the data could be compared, and the importance of the holding pen assessed.

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† Presently Director, Research in Epidemiology and Communications Science Unit, World Health Organization, Geneva, Switzerland.

Two investigations had previously shown that salmonella serotypes in feed could infect swine and be excreted by them. Newell *et al.* (1959) traced infected pigs at slaughter to their farm of origin and found many of the same serotypes in feed ingredients being used on the farms and in pigs being fattened on the farm. In 1960, Smith fed salmonella-free piglets on a known contaminated feed and recovered some of the feed source serotypes from their faeces during life and from their lymph nodes after slaughter. When salmonella-free rations were fed to the remaining animals their excretion stopped.

In this article we describe a longitudinal study conducted on one farm and in one slaughterhouse in Louisiana, where, by changing some of the variables and by the use of serotyping and phage-typing methods, we have attempted to assess the importance of different salmonella sources.

The purpose of our research was twofold. The first was to examine the effect of using pig feed with a decreased level of salmonella contamination in a farm operation. The second was to determine the risk of salmonella infection for each lot of pigs from their environment after leaving the farm and before slaughter. To accomplish these aims six feeding lots of comparable pigs were examined before and after transport, and after slaughter. Samples were also taken from the feed, truck, and abattoir environment before the pigs' exposure to them.

FACILITIES

The pigs used in this experiment were made available by a group in Louisiana growing sugar cane, and raising pigs and feeder calves. Pigs were born and raised to market weight on this well-managed farm. They spent their entire life on concrete. Pens were covered and well drained. They were cleaned daily. No rodent signs were observed in the feeding or storage areas.

Pigs were transported to slaughter in the farm's double-decked open truck with the exception of one lot of eighty that was sent in an open single-decked truck that had previously been used for cattle and not cleaned.

Before the introduction of special feed (low-level salmonella contamination) to the farm all pig feeding, feed mixing, and feed transporting equipment was cleaned with live steam and disinfected with O-syl (Lehn and Fink Products Corp., Bloomfield, New Jersey) at a $1\frac{1}{2}\%$ strength.

The cooperating slaughterhouse was under city inspection only. Sanitation was good in the covered holding pen area, on the kill floor, and in the coolers. When caecal swabs were collected at the time of evisceration, the individual intestines were examined grossly for signs of enteritis. None was observed. Furthermore, no carcasses or carcass parts from study pigs were condemned during the time of these investigations. Carcass sides were washed with cold water, rapidly flowing but not under pressure, and placed directly in the chill room.

FEEDING MATERIALS

The pigs were first fed, after weaning, on a commercial pelleted ration which included meat and bone meal, fish meal, condensed fish solubles, and animal fat.

It was medicated with 50 g. of oxytetracycline per ton. This feed was never sampled.

When pigs reached 50 lb. they were fed on cracked corn and the regular finishing supplement meal made by the same company producing the weaner ration. This contained the same animal origin ingredients as the weaner ration with the exception of animal fat. It, too, was medicated with 50 g. of oxytetracycline per ton.

The special feed used for the study was specially prepared with no animal origin ingredients in it. It was medicated with 80 g. of oxytetracycline per ton. Before the manufacture of each lot of special feed the mill operator 'cleaned' the system by running two tons of cracked corn through it. No attempt was made to assess the usefulness of this measure in the reduction of the contamination level of the special feeds.

SAMPLING METHODS

Pigs and environment

A rectal swab was taken from the pigs in each lot while they were still on the farm, before loading and transport to slaughter. The six lots of pigs were designated as lot I, II A, II B, II C, III A, and III B to correspond with the last lot of feed they received just before slaughter. Following the 4 hr. of transport the pigs were unloaded into abattoir pens. The first three lots (I, II A, II B) were allowed a $1\frac{1}{2}$ hr. period to settle down before rectal swabs were collected from them. The last three lots (II C, III A, III B) were swabbed immediately on arrival. In addition to the swabs taken on arrival, lots I, II B, III A, and III B had rectal swabs taken after an overnight holding period to determine a salmonella build-up. This was just before slaughter.

The truck was sampled before and after transport for lots I, II B and III A, but only after transport for lots II C and III B. The truck used for lot II A pigs was not sampled, either before or after. Pens and their watering troughs were sampled before the entry of each lot of pigs by rubbing floors, gates, fences, and the inside of the trough with sterile swabs.

After slaughter the caecum was incised with a sterile knife and a swab was introduced in such a way that it did not touch the outside of the caecum.

Carcasses were sampled in the chill room by rubbing the swab over the outside and inside of the carcass (both halves) covering the maximum area possible.

All samplings of pigs, environment, and carcasses were done with sterile cotton swabs. These were immediately introduced into fresh tetrathionate for transport and enrichment.

Feed

Feed ingredient samples were collected through the weighing scale inspection door at the time of feed manufacture. They were put in sterile plastic bags until cultured. They were transported and held at ambient temperature if not examined immediately.

Table 1. *Number of isolations from study feeds by type and source*

Lot no. ...	Feed 1 (regular)			Feed 2A (special)	Feed 2B (special)	Feed 2C (special)	Feed 3A (regular)			Feed 3B (regular)		
No. of subsamples ...	29			16	15	21	25			15		
Percentage positive ...	48			6	13	10	36			40		
Source of salmonella type ...	M	S	F	S	S	S	M	S	F	M	S*	F*
anatum	1	2	.	.	.	.	1	.	1	.		
cubana	.	.	.	.	.	1	1	2	.	.		
derby	1	.	.	.	.	.	.	.	.	1		
illinois	2	.	.	.	.	.	.	.	.	.		
infantis	.	.	.	.	.	.	1	.	.	.		
kentucky	.	.	.	.	1	.	.	.	1	.		
livingstone	2	.	.	.	.	.	.	.	.	.		
manhattan	1	.	.	.	.	.	.	.	.	.		
mississippi	.	.	.	.	.	.	.	.	.	1		
newington	.	.	.	.	1	.	.	.	.	.		
newport	2	.	.	.	.	.	.	.	.	.		
oranienburg	.	.	1	.	.	.	.	.	.	.		
poona	.	.	.	.	.	.	.	.	1	.		
senftenberg	2	.	1	.	.	.	.	.	.	.		
simsbury	.	.	.	.	.	2	.	.	.	.		
tennessee	.	.	.	1	.	.	.	4	.	.		
typhimurium copenhagen 3A	1	.	.	.	.	.	.	.	.	.		
Totals	12	2	2	1	2	3	3	6	3	2		

M, meat meal; S, soybean meal; F, fish meal.
* Not sampled—same bin in use as in 3A.

LABORATORY METHODS

Cultural methods were similar to those of Galton (1962). Faecal and environmental samples were placed in tetrathionate broth with 0·001 % brilliant green added, and incubated at 37·5° C. for 18–24 hr. A loopful of broth was streaked onto brilliant green agar (BGS) (Difco) with sulphadiazine added. Feed subsamples of 15 or 30 g. were placed in 50–100 ml. of tetrathionate (Difco) with 3–6 ml. of 1/1000 tergitol added and incubated at 37·5° C. for 72 hr. BGS plates were streaked at 24 and 72 hr. All salmonella strains were screened with a Salmonella H. Antisera Spicer-Edwards Set (Difco) and representative cultures were serotyped in the laboratory of the Louisiana State Board of Health.

All *Salmonella typhimurium* strains were typed by bacteriophage by one of us (Williams) at the Communicable Disease Centre, Atlanta, Georgia in 1963. An incomplete battery of thirteen phages was available and while it was not possible to fit all of the strains to the nomenclature used in that laboratory many consistent patterns were found in each lot of pigs and the strains from their environment. These were useful in demonstrating relationships between strains.

RESULTS

Feed

Three different types of supplement were used. These were designated 1, 2 and 3, and consisted of one, three and two batches respectively. Each batch was manufactured at a different time and differed in the variety of salmonella serotypes recovered and probably the number of salmonella organisms present. The distribution of serotype is shown in Table 1. Feed 1 and 3 were from the same source.

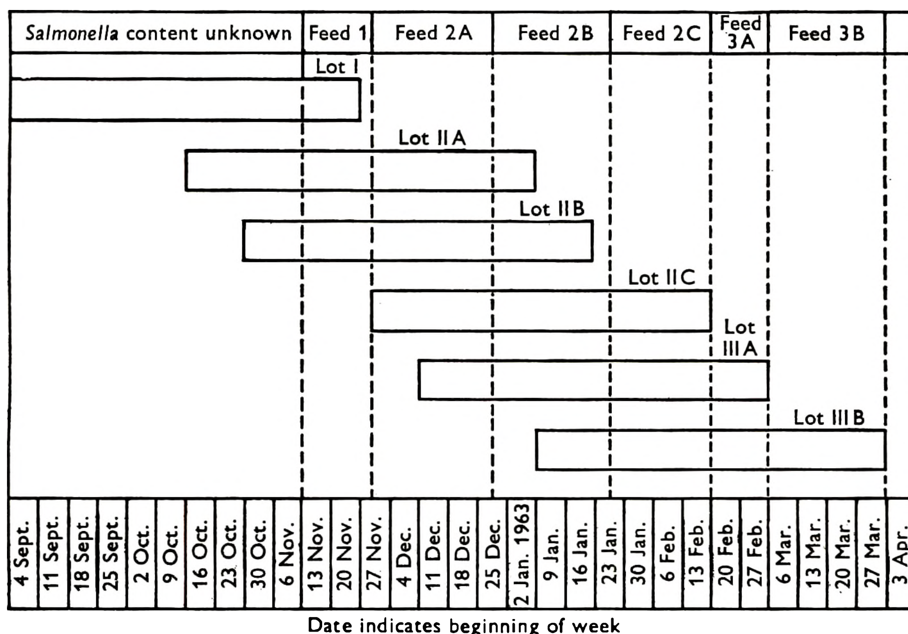

Fig. 1. Feeds consumed by pigs sampled on farm G (11–12-week finishing period). Each lot of pigs described by bars. Changes in feed are indicated by dotted lines.

Forty-one per cent of the subsamples of this regular feed were positive for a salmonella. The same figure for the special feed was 10%. The period of use of these various feeds is shown in Fig. 1 (Williams & Newell, 1967).

Environment and pig samples

A total of 276 rectal swabs were taken from these six lots of pigs while they were still on the farm. In no case was a salmonella isolated from these samples. The additional results of the follow-through sampling of each lot of pigs is shown in Table 2. The greatest number of salmonellas were recovered from lot I pigs and their associated environment after they left the farm. The next highest group was lot IIB pigs. The least number of recoveries came from lot IIIB pigs.

Table 2. *Salmonella recoveries from swine and the environment they were exposed to in marketing*

Source of recovered salmonellas	Pig lot or experiment no. I	IIA	IIB	IIC	IIIA	IIIB
Environmental samples						
Truck before loading	1/10*	.	0/10	.	0/12	.
Truck after transport	3/10	.	1/10	0·10	0/6	3/10
Abattoir pen swabs	12/15	3/8	5/15	0/12	0/10	0/10
Abattoir watering trough swabs	5/5	0/3	2/5	0/5	0/6	0/4
Live pig samples						
Farm rectal swabs	0/50	0/40	0/48	0/40	0/50	0/48
Arrival rectal swabs	26/50	0/40	19/48	0/40	4/50	0/48
Holding rectal swabs†	3/50	—	2/48	—	0/49	0/48
Samples after slaughter						
Caecal swabs	26/49	9/50	17/48	10/50	6/50	1/48
Carcass swabs	4/39	.	8/40	3/40	.	.

* Number of positives over number of samples.
† After 12–19 hr. in abattoir pens.

The results of the sampling of four lots of pigs after the overnight holding period were not as expected. When the pigs were positive on arrival there was a decrease in recovered salmonellas the following morning. This change in excretion has been discussed in a previous paper (Williams & Newell, 1967).

Complex relationships between the salmonella types from the environment and feed and those subsequently recovered in pigs occurred only in lots I and IIB. The abattoir pen and trough water were both contaminated before the entrance of the pigs and these pigs had a $1\frac{1}{2}$ hr. settling-down period before rectal swabbing. This period was apparently long enough to permit passage and excretion of environment types. The results by serotype recovered from lot I pigs or their environment are shown in Table 3. Similar results from IIB pigs have been summarized in Table 4. Lot IIA pigs were the only additional lot exposed to a known contaminated abattoir environment. The abattoir pen contained *S. typhimurium* of phage types 1a and 2a. One isolation of phage type 1a was made from the caecal swabs collected from this lot of pigs.

The results of the prospective sampling of feed and environment were used to assign pig or pig product isolations to one of three sources, food, environment (abattoir), and unknown. Two problems arose in making these determinations which may have affected the accuracy of assignment. The first was the number of

Table 3. *Salmonella serotypes associated with lot I pigs after leaving the farm*

Serotype	Truck before	Truck after	Pen water	Pen	Arrival swab	Caecal swab	Carcass swab
*anatum**	.	.	×	×	×	×	×
archevaleta	.	.	×	.	.	.	.
blockley	.	.	×	.	.	.	.
bredeney	.	.	.	.	.	.	×
cerro	.	.	.	.	×	×	.
*cubana**	.	.	.	.	.	.	.
*derby**	.	.	.	.	.	×	.
*livingstone**	×	×	.	.	×	×	×
montevideo	.	.	.	.	.	×	.
*oranienburg**	.	.	.	.	×	.	.
rubislaw	.	.	.	.	.	×	.
san diego	.	.	×	.	.	.	.
saint paul	.	.	.	.	.	×	.
*senftenberg**	.	×	.	.	.	.	.
typhimurium 2 var.	.	.	×	×	×	×	.
typhimurium NTAP	.	.	.	.	×	.	.

* Serotypes found in feed ingredients fed to these pigs.

Table 4. *Salmonella serotype associated with lot II B pigs after leaving the farm*

Serotype	Truck before	Truck after	Pen water	Pen	Arrival swab	Cecal swab	Carcass swab
typhimurium 1a	.	×	.	.	×	×	.
typhimurium 3a var.	.	.	×	×	×	.	.
typhimurium NTAP	.	.	.	×	×	×	×
typhimurium cope 1 var.	.	.	.	.	.	×	×
*derby**	.	.	.	.	.	×	.
muenchen	.	.	.	.	.	×	.
simsbury	.	.	.	.	.	×	.

* Serotype found in feed ingredients fed to these pigs.

S. typhimurium not typed by available phage (NTAP) that could have been a homogeneous or a heterogeneous group. The second was the presence of *S. anatum* in the feed that lot I pigs consumed and also in the abattoir pen that they were confined to. With nothing similar to a phage typing system to solve this problem the *S. anatum* isolates from lot I rectal and caecal swabs were arbitrarily divided equally into the feed and environment source lists. The results of salmonella recovery by source are summarized in Table 5 and Figs. 2 and 3. Findings from lot I indicate that the contaminated abattoir environment contributed the greatest number of positives of both rectal and caecal swabs. In lot II B pigs the carry-over

of environmental types to the kill floor was not so marked as in lot I pigs according to these methods of ascertainment. When only the pen was contaminated in lot II A no arrival rectal swabs contained a salmonella from any source even though

Table 5. *Percentage recovery of salmonella from rectal and caecal swab samples by most probable origin of salmonella type*

	Swabs	No. of samples	No. positive	Positive (%)	Positive feed type (%)	Positive environment type (%)	Origin unknown (%)
Lot I	Rectal	50	36	72	40*	54*	5·5
	Caecal	49	26	53	33*	60*	8
Lot II A	Rectal	40	0	0	0	0	0
	Caecal	50	9	18	44	11	44
Lot II B	Rectal	48	19	40	21	68	11
	Caecal	48	17	35	38	26	35
Lot II C	Rectal	40	0	0	0	0	0
	Caecal	50	10	20	35	0	65
Lot III A	Rectal	50	4	8	25	0	75
	Caecal	50	6	12	17	0	83
Lot III B	Rectal	48	0	0	0	0	0
	Caecal	48	1	2	0	0	100

* *S. anatum* found in feed and environment—strains from these samples were arbitrarily divided equally into feed and environment source lists.

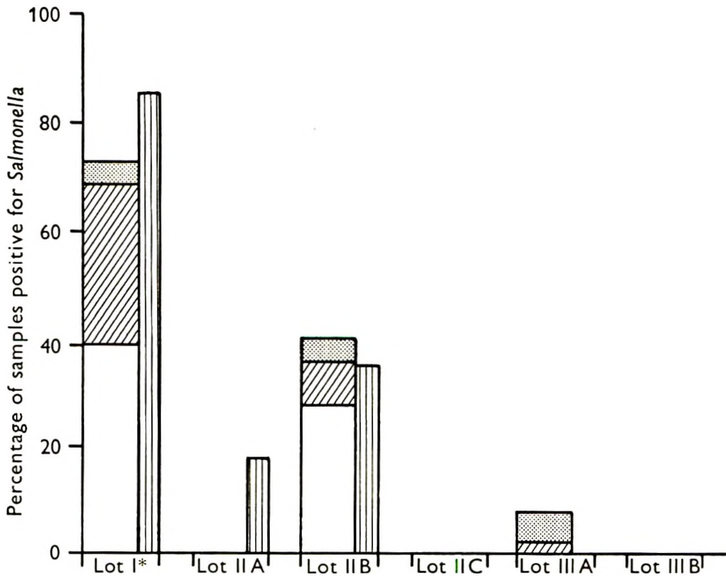

Fig. 2. *Salmonella* recoveries from rectal swab samples taken on pigs' arrival at the plant with proportions of isolations from different sources. (The percentage of environmental positive swabs are shown for comparison.) **S. anatum* found in feed and environment. Plates from which this type only was picked were divided equally between these two sources. ▢ Environment source. ▦ Source unknown. ▨ Feed source. ▥ % of environmental swabs positive.

this group was allowed the settling period; however, following an overnight stay in the pen 11% of the caecal swab recoveries were environmental source types.

Feed source salmonellas were isolated from rectal swabs from lots I, IIB, and IIIA in percentages varying from 21 to 40%. Caecal swab positives attributed to feed sources were recovered in all but the last lot of pigs. The highest of these percentages was 44.

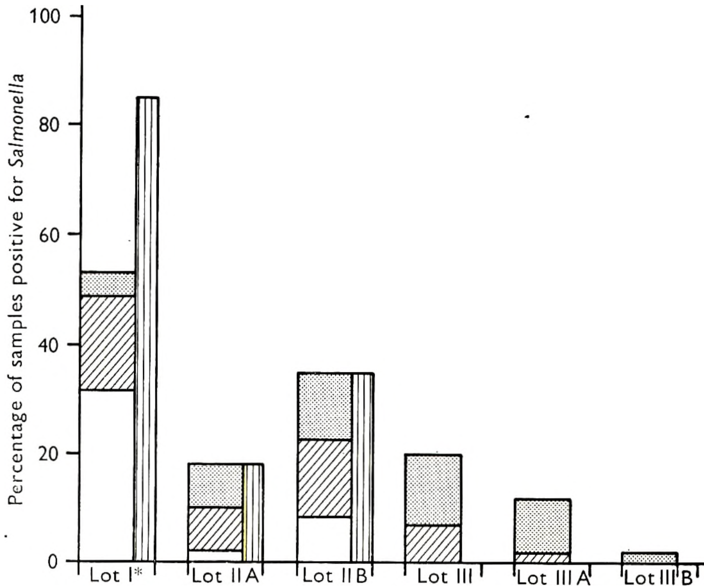

Fig. 3. *Salmonella* recoveries from caecal swab samples with proportions of isolations from different sources (The percentage of environmental positive swabs are shown for comparison.) **S. anatum* found in feed and environment. Plates from which this type only was picked were divided equally between these two sources. ▢ Environment source. ▦ Source unknown. ▨ Feed source. ▥ % of environmental swabs positive.

Table 6. *Recovery of salmonellas from carcass swabs and the probable source of these isolations*

	Carcass samples			Probable origin of carcass isolations		
Date of collection	No. of samples	No. positive	Positive (%)	Feed	Environ-ment	Unknown
(Lot I) 27 Nov. 62	39	4	10	2 (3)*	1 (2)*	0
(Lot IIB) 29 Jan. 63	40	8	20	0	6	2
(Lot IIC) 19 Feb. 63)	40	3	8	1	0	2

* *S. anatum* was recovered from both feed and environment. Isolates could be from either source.

Carcass swabs

During the course of these investigations carcass swabs were taken from lots I, IIB, and IIC. The results of these samplings are shown in Tables 2 and 6. An effort was again made to appraise the probable source or origin of these strains. Feed and the packing plant environment appeared to play equal roles here, though the numbers are very small.

DISCUSSION

The isolation of only seventeen salmonella serotypes from the feed ingredient samples collected in this study must be considered the minimum number of salmonellas present. The number and amount of samples were small and probably not representative. The commercial situations and the limited resources of this investigation restricted the sampling procedures. The very extensive investigation of salmonellas in fish meal by Jacobs *et al.* (1963) indicates the possible deficiencies of our estimate of salmonella content of feed. These workers failed to isolate salmonellas from five 10 g. samples from each of seven bags of fish meal; however, when the entire contents were examined six of the bags yielded salmonellas.

Eight of the seventeen serotypes found in the feed were later isolated from one or more of the rectal or caecal swabs. Only one of the eight (*S. anatum*) was also isolated from the packing plant environment. Our findings were in agreement with Smith (1960) and the earlier work of Newell *et al.* (1959) that indicated that either host selection factors or the infecting dose of salmonella may influence the risk of a pig becoming infected or excreting a salmonella serotype when consuming contaminated feed. Seven serotypes were found in pigs that were not isolated from the six lots of feed or from the abattoir environment. Their source was unknown. The work of Smith (1960) also indicated that the pig, possibly acting as a sort of biological filter, could activate and excrete serotypes from the feed that had not been demonstrated by extensive laboratory examinations. Some of the seven unknown source types may have actually been present in feed ingredients. We would also recall that the first three lots of pigs consumed feed of unknown salmonella content for from 2 to 10 weeks before going on to a sampled feed.

An explanation of why no salmonellas were found in rectal swabs taken on the farm and yet were present after transport has been presented and discussed elsewhere (Williams & Newell, 1967). This change was probably related to stress-induced excretion. The rarity of salmonella excretion on the farm was emphasized in prior experiments using the same farm and the same methods we have described for this study. One pen lot of pigs was taken off antibiotic feeds and rectal-swabbed three times in 72 hr. No salmonellas were isolated from these specimens. At another time five pen lots of pigs (203 total, including just weaned and young pigs) were sampled and only one specimen was positive for a rare serotype (L. P. Williams & K. W. Newell, unpublished data). It was identical with the stock culture being used to check media at the beginning of this study and may have been an error.

The speed with which salmonella serotypes from the pen watering trough were

excreted by entering pigs ($1\frac{1}{2}$ hr exposure) was an unexpected finding. In a British study (Green & Jewell, 1965) food passage rates in pigs were shown to range 24–48 hr. in pigs fed on a standard ration and from 14 to 48 hr. in pigs given senna as a purgative. These animals were not stressed by transit, however, and passage time was measured by the feeding and recovery of polystyrene markers, not by passage of bacteria. There may well be a great difference between food passage rates through farm pigs and the passage of water through market pigs after transport and accompanying stresses.

In two recent controlled studies conducted in the Netherlands, the authors demonstrated that heat-decontaminated feed ingredients and pelleted feeds either prevented or decreased salmonella recoveries from test animals after slaughter (Edel *et al.* 1966; Kampelmacher, Guinée & van Keulen, 1965). We chose exclusion of animal origin products as our method of decreasing the salmonella content of feed because it was the most acceptable to the farm management. They feared the results of heat-treating the supplement as it was not known how it would affect palatability and weight gain. These fears may have been well founded. In the study using heat-decontaminated meals (Kampelmacher *et al.* 1965) it was observed that pigs fed on the decontaminated meal did not have a daily weight gain equal to that of control pigs (510 g. *vs.* 550). Our farm observed no difference in the length of fattening time during the special feeding period. The special feed was also cheaper than the feed regularly used on the farm.

Salmonella recovery rates from both rectal and caecal samples were less in all pigs that consumed the special feed. This was most marked in lots IIC, IIIA and IIIB, that is in pigs that never consumed a known highly contaminated feed until they were well developed or came in contact with a contaminated abattoir environment. It is interesting to note the decrease in caecal swab positives from 10 of 50, to 6 of 50, to a final low of 1 of 48. In experiments with chicks Milner & Shaffer (1952) demonstrated that older chicks were more refractory than young ones to induced *S. typhimurium* infection. If these findings are applicable to young pigs, and if the pelleted weaner ration was salmonella-free, they may account for the unexpected low recovery rate in lots IIIA and IIIB even though these lots consumed regular feed before slaughter.

The pigs fed on special feed for a longer time excreted fewer salmonellas and fewer feed type salmonellas. The majority of the last three lots of pigs did not excrete salmonellas at all on arrival at the plant. They went through a clean plant environment and left it clean for the next group of animals destined to occupy the same pens.

This study demonstrates that there is a build-up of salmonellas at the slaughterhouse and that some of these serotypes come from a contaminated environment. But some of the salmonella excretion and infection found at this time (and later by caecal swab after slaughter) is related to the feed consumed at the farm. These infections may persist, may be the source of infection to other pigs, may be related to abnormal excretion due to stress and other factors, and may in the end contaminate carcass sides. If this is the pattern, there is a chain from animal feed to the kitchen of man.

SUMMARY

A commercial swine fattening ration containing animal origin ingredients was shown to be related to the salmonella excretion of market pigs being sent to slaughter from a well-managed farm. When similar animals from this farm were fed a ration of much lower salmonella content, due to exclusion of ingredients of animal origin, their excretion declined as measured by both rectal and caecal swabs. When subsequent lots of pigs consumed a pelleted weaner ration and the special supplement only, before being exposed to a supplement containing the usual level of contamination, they seemed to be refractory to infection. Probably, this was a function of age at first exposure.

With prospective methods it was possible to show that salmonellas from the abattoir environment could infect pigs, that they would excrete them within a very short time, and that organisms from this source could be demonstrated at slaughter and shown to be a cause of carcass contamination.

While these findings support the view that the build-up of salmonellas in pigs is by contact with a contaminated environment, they indicate that the primary source of the contamination is most probably the salmonella-excreting pig which has consumed contaminated feed ingredients on its farm of origin.

If the great majority of pigs went to slaughter salmonella-free they would not serve as a source of infection to other pigs being sent to slaughter. Their intestinal contents could not contaminate carcass sides in the slaughtering process. This should help to prevent the contamination with salmonellas of the food preparation area of both homes and commercial eating establishments.

We extend sincere thanks to L. A. Frey and Sons, Inc., New Orleans, Louisiana, and Graugnard Farms, St James, Louisiana, who demonstrated their interest in these investigations by letting us use their facilities. We are especially grateful for the work and support of Edmund G. Guillot, then livestock manager for the latter-named organization. We also appreciated the work of Dr George H. Hauser, Director, Louisiana State Board of Health, for arranging the serotyping of salmonella strains from this investigation. Figure 1 is reproduced by permission from *The American Journal of Public Health*, **57**, 468.

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Inhibition by pyrimidine analogues of the synthesis of folic acid by trachoma agents

BY P. REEVE* AND JANICE TAVERNE
M.R.C. Trachoma Research Unit,
Lister Institute of Preventive Medicine, London, S.W. 1

AND S. R. M. BUSHBY
Wellcome Research Laboratories, Beckenham, Kent

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INTRODUCTION

The *Chlamydia* may be divided into two groups by their reaction to sulphonamides (Moulder, 1964). One group comprises agents like psittacosis and meningopneumonitis which are resistant to sulphonamides and which utilize an exogenous source of folic acid. The other group comprises agents like those of mouse pneumonitis, lymphogranuloma venereum and trachoma whose growth is inhibited by sulphonamides, indicating that they synthesize their own folic acid.

Trimethoprim, a derivative of 2,4-diaminopyrimidine, inhibits the growth of many species of bacteria (Bushby & Hitchings, 1968; Hitchings & Burchall, 1965; Bushby & Barnett, 1967). It is effective *in vitro* and *in vivo* and has been used therapeutically (Noall, Sewards & Waterworth, 1962; Cooper & Wald, 1964; Drew, Hughes & Jenkins, 1967; Drew, Hughes, Fowle & Cassell, 1967; Czonka, 1967). This compound, which competes with folic acid, inhibits dihydrofolate reductase. In combination with sulphonamides it has a strong potentiating action, as a consequence of the sequential blockade of the biochemical pathway that leads to the synthesis of coenzyme F.

Since the trachoma agent apparently synthesizes folic acid, we tested its susceptibility to trimethoprim and to the related 2,4-diaminopyrimidine derivative pyrimethamine, alone and in conjunction with a sulphonamide, and we present evidence for the existence of a dihydrofolate reductase in the trachoma agent.

METHODS

Trachoma and inclusion conjunctivitis (*TRIC*) *agents*

TRIC agents are named according to the system proposed by Gear, Gordon, Jones & Bell (1963). The abbreviations used in this paper are given in brackets. Fast-killing variants are suffixed *f* (Taverne, Blyth & Reeve, 1964*a*).

TRIC//China/Peking-2/OT (T'ang, Chang, Huang & Wang, 1957) (PK-2) and its variant PK-2*f* (T'ang).

* Present address: Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, London, W. 12.

The fast-killing variant (HAR-2*f*) of strain TRIC//SAU/HAR-2/OT (Murray *et al.* 1960).

TRIC//GB/MRC-4/ON (Jones, 1961) (MRC-4).

TRIC//WAG/MRC-1/OT (Collier & Sowa, 1958) (MRC-1).

TRIC//WAG/MRC-062/OT (Taverne, Blyth & Reeve, 1964*b*) (MRC-062).

Strains were grown in yolk sacs of 7-day chick embryos kept at 35° C. Suspensions were made and stored, and titrations were done as previously described (Taverne *et al.* 1964*a*). Dilutions were made in phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) without streptomycin.

Reagents

Trimethoprim lactate [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine] was dissolved in distilled water and sterilized by autoclaving.

Pyrimethamine [2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine] was dissolved in distilled water by the addition of a few drops of isethionic acid and was sterilized by autoclaving.

Leucovorin calcium (Lederle) [5-formyl-5,6,7,8-tetrahydrofolic acid] was dissolved in PBS and sterilized by filtration.

Sulphafurazole was dissolved in distilled water with the aid of a few drops of 0·1 N-NaOH and was sterilized by autoclaving.

These compounds were tested at various concentrations, prepared from stock solutions (usually containing 2 mg./ml.) by making serial dilutions in distilled water, PBS, or in a given suspension of a trachoma agent.

Tests for inhibition

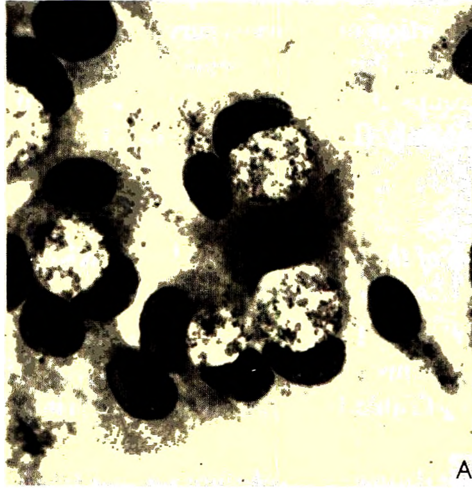
In cell culture

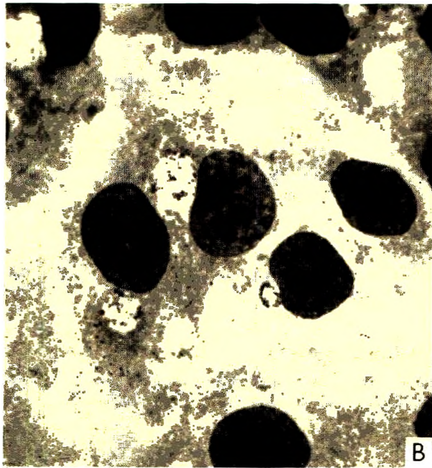
HeLa cell monolayers in Leighton tubes were inoculated with 1 ml. of medium 199 containing a concentration of strain HAR-2*f* calculated to form about ten inclusions per microscope field, together with various concentrations of inhibitor or inhibitors. Cultures were incubated at 37° C. for 48 hr., fixed and stained with Giemsa or iodine for inclusion counts (Furness, Graham & Reeve, 1960). The number of inclusions formed in the presence of inhibitor was expressed as a percentage of those in the controls inoculated with HAR-2*f* only.

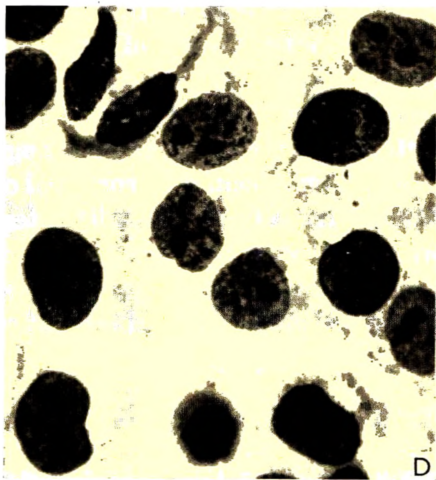
In chick embryos

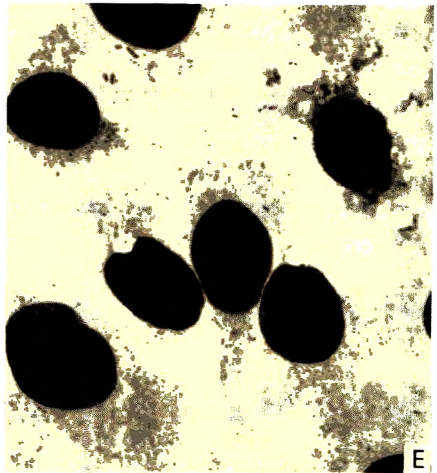
Groups of 7-day chick embryos were inoculated with a single dilution of a suspension of a trachoma agent containing about 10^5 LD 50, calculated to kill the embryo in 5–7 days, and with the same dilution containing various concentrations of the compound to be tested. Control tests of the toxicity of the compounds for chick embryos in the absence of the trachoma agent were also included.

Volumes of 0·5 ml. were inoculated into the yolk sac and the eggs were incubated at 35° C. in an atmosphere of 65 % relative humidity. Eggs were candled daily until tests were terminated on the 13th or 14th day after inoculation. The specificity of death, when doubtful, was checked by the examination of yolk sac smears stained with Giemsa for the presence of elementary bodies. The action of the









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compounds on the growth of the trachoma agent was determined in two ways: first, in terms of the proportion of embryos surviving infection in the presence of different concentrations of inhibitor, and secondly, in terms of prolongation of the mean survival time of groups of embryos compared with that of the control group receiving trachoma agent only (Lin & Moulder, 1966).

RESULTS

Inhibition of the trachoma agent by trimethoprim and sulphafurazole in cell culture

Concentrations higher than 10 μg./ml. of sulphafurazole or of trimethoprim decreased the number of inclusions formed by strain HAR-2*f* in HeLa cell monolayers by more than 50 % (Table 1). Concentrations of trimethoprim greater than

Table 1. *Inhibition by sulphafurazole and trimethoprim of strain HAR-2f grown in HeLa cells*

	Inhibition* (%)	
μg./ml.	Sulphafurazole	Trimethoprim
500	59·5	N.D.
100	N.D.	73·6†
50	53·7	N.D.
10	45·0	55·9
2·5	33·0	15·0

* The number of inclusions in the test cells expressed as a percentage of the number in the control.

† Cells in poor condition, probably because of the toxic effect of the drug.

N.D. = Not done.

100 μg./ml. were toxic for the cells. Over a given range, the percentage inhibition was roughly proportional to the concentration of inhibitor present. It was, however, difficult to obtain reliable inclusion counts. The total number of inclusions did not change very much over a wide range of drug concentrations, but the appearance of the inclusions varied greatly. Both drugs caused similar gross changes in the morphology of the inclusions (Plate 1), and the extent of the change depended on concentration. With higher concentrations, inclusions consisted of small, empty vacuoles which, nevertheless, indented the cell nucleus; with intermediate concentrations, the vacuoles were bigger but contained only a few elementary bodies; at lower concentrations their morphology appeared to be almost normal. However, the change with drug concentration in the size and content of

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EXPLANATION OF PLATE

Changes in the morphology of inclusions formed by the HAR-2*f* strain of trachoma in HeLa cells in the presence of different amounts of trimethoprim, × 640. A = Control, in absence of inhibitor. B = 0·1 μg. per ml. trimethoprim. C = 1·0 μg. per ml. trimethoprim. D = 10 μg. per ml. trimethoprim. E = 100 μg. per ml. trimethoprim.

the inclusions was so gradual that it was not possible to decide on the end-point of this effect.

On the assumption that inhibition of multiplication took place at an early stage in the growth cycle, before the synthesis of the glycogen matrix, an attempt was made to estimate the effect of the drugs by staining inclusions with iodine 48 hr. after infection. However, although inclusions were abnormal when stained with Giemsa in the presence of concentrations of sulphafurazole ranging from 10 to 0·25 μg./ml., they still stained with iodine.

Inhibition tests on combinations of various concentrations of sulphafurazole and trimethoprim showed that at certain concentrations the combined effect of the two drugs was at the least additive (Fig. 1). As with each drug alone, inclusion counts of the same order were obtained over a wide range of concentrations although the appearance of the inclusions changed; but since this change had no precise end-point, it could not be taken into account in assessing the degree of potentiation.

It was clear from the absence of elementary bodies in inclusions stained with Giemsa that both drugs inhibited the formation of infective agent. To measure the suppression of multiplication we investigated their action in the egg, technically a more convenient system.

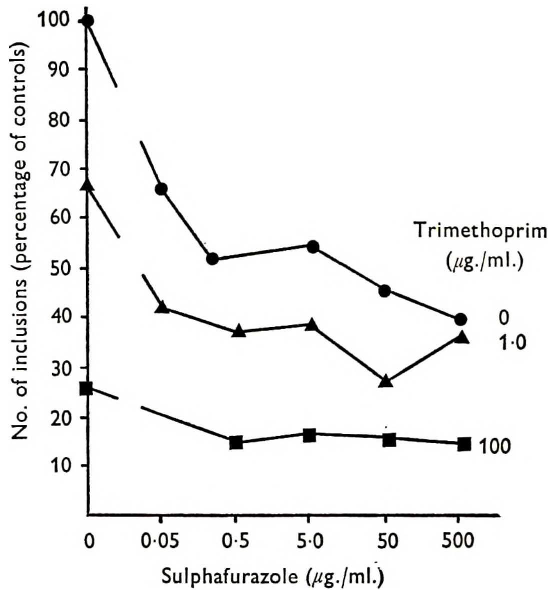

Fig. 1. The effect of two concentrations of trimethoprim on the numbers of inclusions formed by HAR-2*f* in HeLa cells in the presence of various concentrations of sulphafurazole. ●, No trimethoprim; ▲, 1·0 μg./ml. trimethoprim; ■, 100 μg./ml. trimethoprim.

Inhibition of trachoma agents by trimethoprim and sulphafurazole in chick embryos

Trimethoprim inhibited the growth of three slow-killing strains (MRC-4, MRC-1 and PK-2) and the one fast-killing strain of TRIC agent tested (T'ang), but on a weight basis it was about 50 times less active than sulphafurazole. Even at a concentration of 5000 μg. per egg it failed to inhibit one strain, MRC-062,

although the strain was as susceptible to sulphafurazole as other strains. When the increase in mean survival time of groups of infected embryos was plotted against the concentration of inhibitor they received, expressed on a logarithmic scale, a direct relationship was observed (Fig. 2). Doses of sulphafurazole greater than about 300 μg. per embryo prolonged survival until most of the embryos began to hatch. With trimethoprim, the mean survival time was never sufficiently prolonged for the embryos to hatch; at doses above about 5000 μg. the embryos died sooner than controls given no trimethoprim, presumably because of the toxicity of the drug. The percentages of chick embryos infected with a lethal dose of MRC-4 and T'ang surviving infection in the presence of various concentrations of sulphafurazole or trimethoprim are given in Table 2.

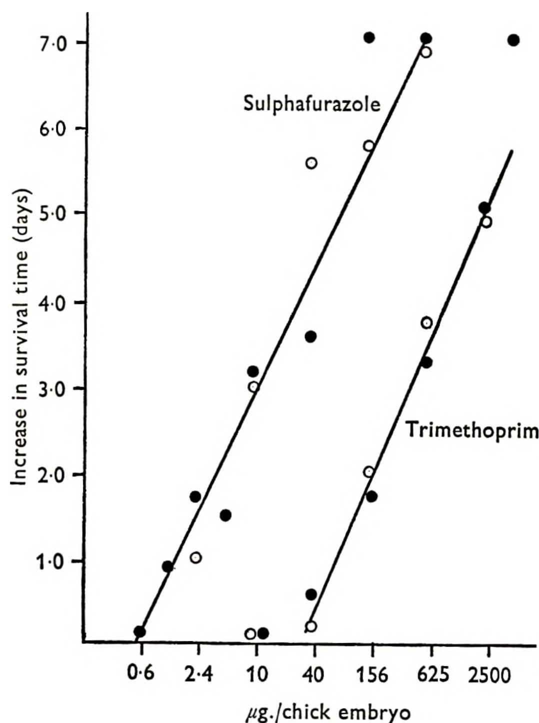

Fig. 2. Effect of sulphafurazole and trimethoprim on the survival of chick embryos inoculated with strain T'ang, ○, or strain MRC-4, ●. Chick embryos hatched if the survival time was extended beyond about 7 days.

Tests for potentiation of the activity of sulphafurazole by trimethoprim

Groups of embryos were inoculated with a lethal dose of either MRC-4 or T'ang, together with concentrations of sulphafurazole and trimethoprim in the range in which there was a linear relationship between concentration and prolongation of mean survival time.

From the average day of death (Tables 3 and 4) prolongation of mean survival time was determined. For each strain, the increase in mean survival time was plotted in terms of the effect of concentrations of trimethoprim on the activity of sulphafurazole. As an example, results obtained with strain MRC-4 are given

Table 2. *Percentage survival of chick embryos infected with strains MRC-4 or T'ang in the presence of graded amounts of sulphafurazole or trimethoprim*

Dose (μg.) per chick embryo	Sulphafurazole		Trimethoprim	
	MRC-4*	T'ang*	MRC-4*	T'ang
0	0	0	0	0
0·7	0	0	N.D.	N.D.
2·5	11	0	0	N.D.
10	65	16	0	0
40	80	45	0	12
156	100	78	0	0
625	100	94	29	28·5
2,500	100	90	84	84
5,000	N.D.	N.D.	100	N.D.
10,000	N.D.	N.D.	Toxic for chick embryos	

* Mean values for two tests.
N.D. = not done.

Table 3. *The average day of death of groups of chick embryos infected with strain T'ang in the presence of graded amounts of sulphafurazole and trimethoprim*

Trimethoprim per chick embryo (μg.)	Sulphafurazole per chick embryo (μg.)					
	0	0·7	2·5	10	40	150
0	5·0	4·5	6·3	8·8	9·7	10·0
10	5·1	4·7	6·8	8·6	11·0	11·0
40	5·1	5·9	8·2	10·6	10·4	9·1
150	6·6	7·3	9·6	8·7	8·7	11·0
625	8·0	9·6	10·5	9·1	9·1	8·5

Table 4. *The average day of death of groups of chick embryos infected with strain MRC-4 in the presence of graded amounts of sulphafurazole and trimethoprim*

Trimethoprim per chick embryo (μg.)	Sulphafurazole per chick embryo (μg.)				
	0	0·7	2·5	10	40
0	6·8	6·9	8·5	10·0	> 14
10	7·4	6·9	8·5	10·0	> 14
40	7·0	7·1	8·4	10·3	> 14
150	8·5	9·6	10·5	13·7	> 14
625	9·8	11·2	9·6	> 14	> 14

(Fig. 3). From the lines fitted to these plots, the minimum concentration of sulphafurazole extending the mean survival time by an arbitrary period of 3 days in the presence of graded concentrations of trimethoprim was read off; the minimum effective concentration of the drugs acting alone was similarly estimated from Fig. 2

(Table 5). (The selection of such an arbitrary period is valid since the lines in Fig. 2 are parallel.) These values (cols. 1 and 2) were expressed as percentages of the minimum effective concentration of each drug acting alone (cols. 3 and 4). For example, 9·8 μg. of sulphafurazole prolonged survival of embryos infected with the

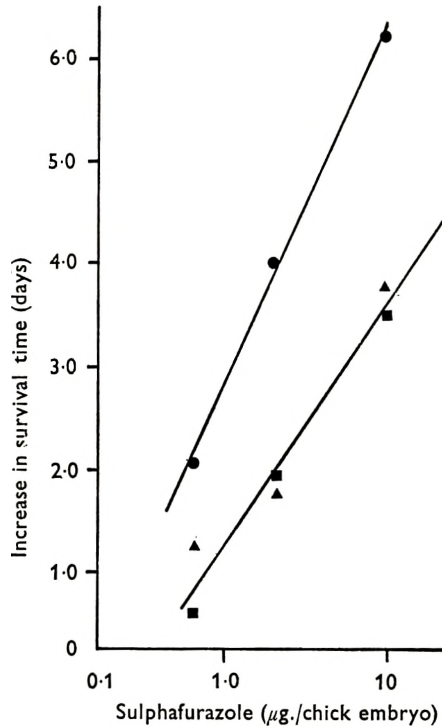

Fig. 3. The prolongation of mean survival time of chick embryos inoculated with MRC-4 and various combinations of sulphafurazole and trimethoprim. ● = 150 μg. trimethoprim; ▲ = 40 μg. trimethoprim; ■ = 10 μg. trimethoprim.

Table 5. *Combinations of trimethoprim and sulphafurazole prolonging the survival of chick embryos infected with strain T'ang or MRC-4 by 3 days*

			Percentage concentration effective in absence of the other drug		
Strain	Tri-methoprim μg./embryo	Sulpha-furazole μg./embryo	Tri-methoprim	Sulpha-furazole	Sum of percentages
T'ang	0	9·8	0	100	100
	10	9·8	2	100	> 100
	40	7·2	8	74	82
	150	3·2	30	33	66
	500	0	100	0	100
MRC-4	0	9·8	0	100	100
	10	8·0	2	80	82
	40	8·0	8	80	88
	150	1·6	30	17	47
	500	0	100	0	100

T'ang strain for 3 days in the absence of trimethoprim; in the presence of 150 μg. of trimethoprim only 3·2 μg. (33 % of 9·8) was required. Potentiation is assumed to have occurred when the sum of the pairs of percentages for each combination of drugs is less than 100. Only with 150 μg. of trimethoprim was the sum of these percentages significantly less than 100, indicating potentiation, which was, however, not very great.

Reversal of trimethoprim inhibition by calcium leucovorin

Trimethoprim could inhibit the multiplication of TRIC agents, by blocking a reaction essential to a synthetic process outside the folic acid cycle. If it acts solely by blocking the reduction of folic acid to citrovorum factor, addition of this factor should reverse the inhibition.

Various concentrations of calcium leucovorin were inoculated into the yolk sacs of groups of chick embryos infected 1 hr. previously with a lethal concentration of the T'ang strain and graded concentrations of trimethoprim. Calcium leucovorin

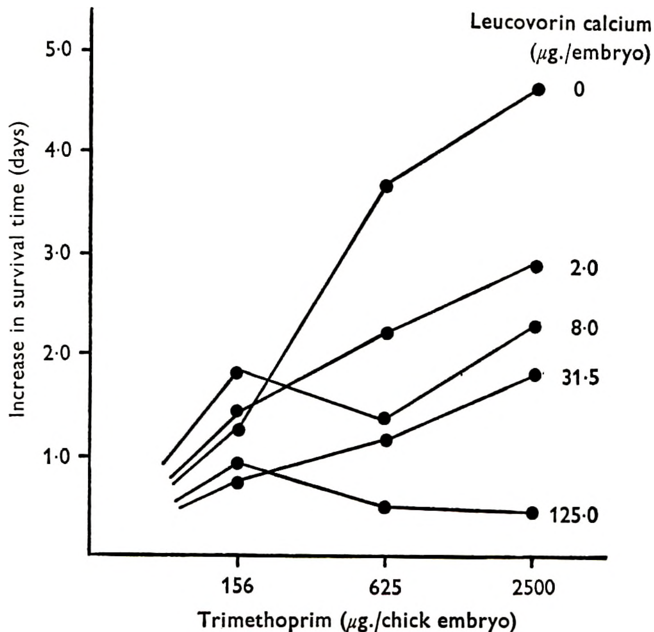

Fig. 4. The effect of graded concentrations of calcium leucovorin on the inhibitory action of trimethoprim against the T'ang strain of trachoma multiplying in the chick embryo yolk sac.

itself was not toxic and did not influence the multiplication of the T'ang strain. The prolongation of survival time of the embryos so treated compared with controls infected with T'ang and given calcium leucovorin only was plotted against log concentration of trimethoprim (Fig. 4). The inhibition by all concentrations of trimethoprim tested did not occur in the presence of 125 μg. per embryo of calcium leucovorin. All concentrations of leucovorin tested reversed the protection obtained with trimethoprim to some extent, in proportion to concentration.

Inhibition by pyrimethamine

The susceptibility of the trachoma agent to another inhibitor of folate synthesis was tested in groups of chick embryos inoculated with a lethal dose of the T'ang strain or MRC-4 and given various concentrations of the antimalarial compound pyrimethamine. Concentrations of 500 μg. per embryo or more were toxic, killing nearly all the embryos within 2–3 days of administration. In the range 100–500 μg. per embryo, pyrimethamine had little effect on the mean survival time of embryos infected with MRC-4, but significantly prolonged that of those infected with the T'ang strain. The prolongation in survival time was proportional to dose, expressed logarithmically, but the relation could be expressed only over a small range of concentrations of the drug because of its toxicity (Figs. 5, 6). In one experiment, groups of at least thirty eggs were inoculated with a lethal dose of strain MRC-4 or T'ang, alone or with 250 μg. of pyrimethamine per embryo, and the difference

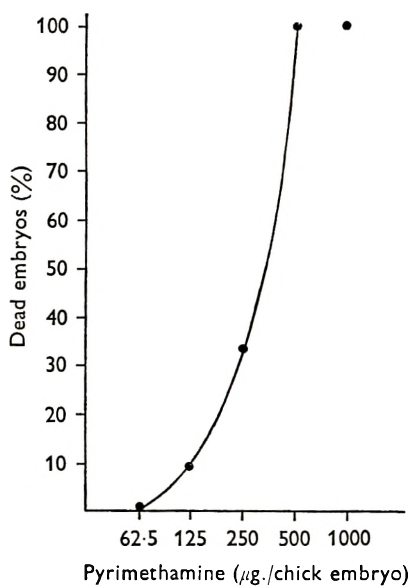

Fig. 5

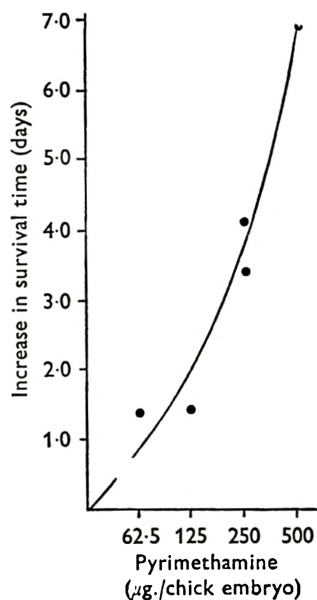

Fig. 6

Fig. 5. The toxic effect of pyrimethamine on the chick embryo. Deaths were counted within 6 days of inoculation.

Fig. 6. The prolongation of mean survival time of chick embryos infected with the T'ang strain in the presence of graded concentrations of pyrimethamine.

in mean survival time was analysed statistically. With strain T'ang the mean survival time was prolonged 4·1 days and this difference was highly significant ($P = < 0{\cdot}01$); with strain MRC-4 the prolongation was 1·4 days, which was only just significant ($P = 0{\cdot}04$).

DISCUSSION

Trimethoprim inhibited the growth of several strains of trachoma agent and the one strain tested of inclusion conjunctivitis. Although there were some differences in the susceptibility of various strains to both pyrimidine analogues, no clear

pattern emerged. Resistance to one was not necessarily accompanied by resistance to the other; for instance, strain MRC-4 was inhibited by trimethoprim but not by pyrimethamine, and preliminary experiments indicated that strain MRC-062, which was resistant to trimethoprim, was sensitive to pyrimethamine. Again, sensitivity did not appear to be related to virulence for the chick embryo. Thus, although the more virulent strain T'ang was inhibited by pyrimethamine whereas the less virulent strain MRC-4 was not, tests on two other less virulent strains indicated that PK-2 was sensitive whereas MRC-1/OT apparently was not.

In eggs, inhibition of growth of the trachoma agent by trimethoprim was most clearly demonstrable in terms of the prolongation of mean survival time of groups of embryos inoculated with a given dose of agent and various concentrations of drug. There was no such clear relation between dose and the proportion of embryos protected against death; only the higher concentrations of trimethoprim protected embryos and these concentrations were themselves toxic for the embryos.

Cell cultures did not provide a useful alternative test system because trimethoprim changed the morphology of inclusions and made counting difficult. Furthermore, only fast-killing variant strains can easily be titrated in cell culture. Since they differ in many respects from freshly isolated strains and may differ in their sensitivity to drugs, it was considered important to use a method of titration applicable to all strains.

The trachoma agent multiplies in mouse lungs (Graham, 1965) but in mice trimethoprim did not inhibit infection by either the slow-killing strain MRC-1 or the variant HAR-2*f* (S. R. M. Bushby, and M. Barnett, unpublished results) nor did it potentiate the activity of sulphafurazole. However, even concentrations as high as 2·0 mg. of sulphafurazole per mouse, the highest dose tested, protected only 80 % of the mice; the same dose per unit body weight protected nearly 100 % of chick embryos. Because doses of agent sufficient to kill by infection all the mice inoculated often kill by direct toxicity, the mouse lung test was considered to be unsatisfactory.

Since chlamydia sensitive to sulphonamides are known to synthesize folic acid (Moulder, 1964), it was not unreasonable to suppose that trimethoprim inhibited the multiplication of the trachoma agent by preventing the utilization of folic acid. Since the action of the drugs appeared to be reversed by calcium leucovorin, we conclude that these agents most probably contain a dihydrofolic acid reductase. Burchall & Hitchings (1965) demonstrated the binding *in vitro* of 2,4-diamino pyrimidines, including trimethoprim, to dihydrofolate reductases isolated from *Escherichia coli*, *Staphylococcus aureus* and *Proteus vulgaris*. There was a strong correlation between the degree of binding by a particular reductase and the capacity of the drug to inhibit the micro-organism from which it was derived. A lesser degree of binding was exhibited by reductase isolated from mammalian cells. Thus the therapeutic value of trimethoprim depends on its differential action on enzymes of parasite and host.

Although in our system trimethoprim was toxic for the host, whether chick embryo or HeLa cells, inhibition of the trachoma agent was demonstrable by concentrations of inhibitor that were not toxic for the host. The closeness of the

minimum effective therapeutic dose to the toxic dose suggests that the difference between the binding of trimethoprim to reductases of these agents and to the reductases of the chick embryo was less than the differences between the binding of the enzymes of susceptible bacteria and mammalian tissues. Although the pyrimidine somewhat enhanced the activity of a sulphonamide its effect was slight compared with potentiation against bacteria and susceptible protozoa (Clarke, 1962; Eyles & Coleman, 1953; Rollo, 1955). The low degree of potentiation may be due to poor and incomplete binding of the reductases of the trachoma agent, so that some enzyme is free to deal with folate that has escaped the blocking effects of the sulphonamide.

The structure of the antimalarial compound pyrimethamine resembles that of trimethoprim, and it acts against the malarial parasite by inhibiting the utilization of folic acid. Although weight for weight it was more active against the trachoma agent than trimethoprim, it was considerably more toxic for the chick embryos and its action was demonstrable only over about an eightfold range of concentration. Evidence that antimalarial drugs are active against ophthalmic trachoma has not yet been sought, but the laboratory findings suggest that it is potentially able to affect the disease in the field, either alone or synergistically with other drugs. This factor should be considered when evaluating the incidence of trachoma or planning vaccine or drug trials in areas where malaria is endemic and diaminopyrimidines are in general use. It may be profitable to investigate the activity against trachoma of other folic acid antagonists of this type.

SUMMARY

Trimethoprim, a 2,4-diaminopyrimidine derivative which inhibits the growth of some bacteria by interfering with folic acid synthesis, inhibited the growth of several strains of the trachoma agent. Inhibition was most clearly demonstrated by measuring prolongation of mean death time of groups of chick embryos inoculated with a single lethal dose of agent. Over a certain range, prolongation was proportional to the logarithm of concentration of inhibitor; higher concentrations were toxic for the embryo. On a weight basis, trimethoprim was not as active as sulphafurazole. Inoculation in conjunction with sulphafurazole resulted in slight potentiation of activity. A related pyrimidine derivative, the antimalarial drug pyrimethamine, also significantly inhibited the growth of one strain of trachoma.

In cell culture, trimethoprim decreased the number of inclusions formed by a suspension of the trachoma agent and induced morphological changes in the inclusions similar to those caused by sulphafurazole.

Inhibition of the growth of the trachoma agent in the chick embryo was reversed by leucovorin calcium. It is concluded that, as with bacteria, the drug acts by blocking the folic acid cycle and that the trachoma agent most probably contains a dihydrofolate reductase.

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Further studies on the development of a live oral cholera vaccine

BY P. BHATTACHARYA* AND S. MUKERJEE

Department of Microbiology, Indian Institute of Experimental Medicine, 4, Raja Subodh Mullick Road, Calcutta-32

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The inadequacy of the injectable cholera vaccine presently in use for prophylaxis against cholera has been brought out in recent field trials. The *W.H.O. Cholera Information* no. 4 (1965) in its editorial on the results of these trials observed that a safe and effective vaccine had still to be developed for obtaining a high degree of protection against cholera in man. While one way of improving the vaccine would be to lower the toxicity without decreasing the protective effect, it was suggested that more effective immunization might be possible by routes other than the parenteral.

Mukerjee (1963) studied the possibility of developing a live cholera vaccine for administration by the oral route and obtained evidence, both by *in vitro* and *in vivo* experiments, indicating the suitability for this purpose of an El Tor vibrio strain isolated from the Middle East or from water sources in Calcutta in the absence of cholera. In a subsequent publication Mukerjee (1965) reported the stability of the avirulent character of the proposed vaccine strains on repeated propagation in animals or artificial lysogenization with phages from virulent cholera cultures. These strains have been further examined with special reference to their ability to multiply in the intestine, which is considered a prerequisite for stimulating the development of local cellular immunity (Suter & Ramseir, 1964). These studies as well as the results of further work on the pathogenicity and immunizing value of the vaccine strains and the nature of immunity produced in laboratory animals by live vaccination by oral or intra-intestinal routes are reported in this communication.

MATERIALS AND METHODS

Vibrio strains

The *Vibrio cholerae* strains used in these experiments had been isolated from patients in India. The two El Tor strains proposed to be used as oral vaccine are ME-7 isolated in a Middle East country and EW-6 from a water source in Calcutta in 1958 (Mukerjee, 1963). These strains were used in the present series of tests also. They will be referred to as 'vaccine strains'. The *V. eltor* (case) strains had been isolated in infected areas from either patients or carriers. All *V. cholerae* strains and the *V. eltor* (case) strain no. HK 130 belonged to the Inaba serotype, the rest of the

* Present address: University of California, Department of Bacteriology, Los Angeles, California 90024, U.S.A.

El Tor strains being Ogawa. *V. eltor* strains in this series were haemolytic at the time of test and non-susceptible to group IV cholera phage, while the *V. cholerae* strains were non-haemolytic and lysable by this phage.

Animals

The rabbits used in these experiments were obtained from local suppliers and were heterozygotic. Some of the infant rabbits, however, were bred in our animal house from pure stock obtained from the Haffkine Institute, Bombay, and Central Drug Research Institute, Lucknow.

Pathogenicity test in adult rabbits

The test was carried out according to the method of De & Chatterjee (1953) and has been described in detail by Mukerjee (1963).

Pathogenicity test in infant rabbits

The test was carried out according to the method of Dutta & Habbu (1955). A 3 hr. broth culture (diluted in saline or undiluted) containing approximately $2{\cdot}5 \times 10^4$–10^9 viable vibrios was injected in the proximal part of the small intestine. As a rule, higher doses were used in tests with apathogenic strains.

Protection test in adult rabbit

A 3 hr. broth culture of ME-7 containing approximately 5×10^8 viable cells in 0·5 ml. was injected into the proximal part of the small intestine under ether anaesthesia. After 3–6 days the immunity of the rabbit to challenges with *V. cholerae* and *V. eltor* (case) strains was tested in ligated intestinal loops.

Protection test in infant rabbit

Using a rubber catheter, infant rabbits were given by mouth 100, 1000 and 10,000 viable vibrio cells from a 3 hr. culture of the ME-7 strain on the 3rd, 5th and 7th days respectively after birth. The rabbits were challenged intra-intestinally between the 10th and 12th day with virulent cultures of an El Tor (case) strain.

Tests for stability of the avirulent character of vaccine strains after animal passage

The ME-7 and EW-6 strains were used in two different series of experiments. These strains were serially propagated and re-isolated from the ligated intestinal loops of adult rabbits and intestinal tract of infant rabbits. In each series propagation was repeated ten times and the virulence of the strains was carefully examined for any alteration.

Tests on the multiplication of the vaccine strains in adult rabbits

ME-7, GS 1/65 and GS 9/65 were used as representative strains of apathogenic *V. eltor*, *V. cholerae* and pathogenic *V. eltor* respectively. Three-hour growth of a vibrio strain diluted in nutrient broth was injected into the intestinal loop of an

adult rabbit. Viable counts were made from samples used for the injection. Twenty-four hours after injections the contents of the loops were washed out in saline and a viable vibrio count of each sample was made after plating.

Tests on the multiplication of the vaccine strain in infant rabbits

ME-7 and GS 9/65 were taken as representative apathogenic and case El Tor strains. Half ml. quantities of diluted 3 hr. growth of the strains in nutrient broth were injected into the proximal part of the small intestine under ether anaesthesia. Viable counts of the vibrio cells per ml. of the samples injected were made. After 24 hr. the reactions in the gut were noted. The contents of the small and large intestine were washed out in a fixed volume of normal saline and the viable vibrios in the sample counted after plating on nutrient agar. The total viable cells found in the intestinal contents as compared with the number injected indicated the rate of multiplication.

Tests on multiplication of the vibrios in gut of adult rabbit after intra-intestinal immunization

Adult rabbits were immunized by injection, into the upper part of the small intestine, of 2·5 ml. of a saline suspension of an 18 hr. growth of ME-7 containing about $2{\cdot}0 \times 10^{11}$ viable cells. On the eighth or ninth day of immunization known numbers of viable cells of *V. cholerae* (569B or GS 1/65) and *V. eltor* (pathogenic and apathogenic types) were injected into three isolated loops of small intestine of equal length in each rabbit. After 4, 8 and 24 hr. or in some experiments only after 24 hr. the rabbits were killed, reaction in the loops noted and viable counts of the loop contents were made. With each immunized animal one normal animal was generally inoculated with the same number of vibrios as a control.

RESULTS

Pathogenicity of the vaccine strain

The degree of pathogenicity for laboratory animals of the Middle East and water El Tor strains as compared with that of *V. cholerae* and *V. eltor* strains isolated from cholera cases was retested in an extended series of experiments. The results are given in Tables 1 and 2. It may be seen from Table 1 that all *V. cholerae* and *V. eltor* (case) strains gave rise to inflammatory reactions in all ligated intestinal loops of the thirty rabbits tested. On the other hand, all loop tests of ME-7 and EW-6 strains in 34 rabbits failed to produce any reaction excepting in one instance where injection of the EW-6 strain caused slight swelling. Table 2 shows that all infant rabbits receiving cultures of *V. cholerae* and *V. eltor* (case) strains showed a positive reaction, while no reaction was noted in 4 rabbits injected with EW-6 and only 1 of the 19 rabbits receiving ME-7 showed slight distension of the large gut with fluid.

Mukerjee (1963) reported that the typical reaction of profuse diarrhoea and extreme dehydration described by Dutta & Habbu (1955) could not be found in infant rabbits of the heterozygotic type obtained from suppliers in Calcutta.

Identical observations were made in the present series even when litters of pure-bred stock raised in the laboratory were used. A positive reaction was accordingly judged by the death of the animal and typical distension of the large intestine with fluid.

Table 1. *Tests for gut inflammatory reaction in normal adult rabbits*

Vibrio type	Strain no.	Area of isolation	Total no. of tests	No. of tests showing positive reactions
V. cholerae	H 86/62	Calcutta	5	5
	H 96/64	Calcutta	4	4
V. eltor (case)	Baroda 6/64	India	2	2
	Baroda 7/64	India	1	1
	Bg 79/64	India	6	6
	Bom 1/65	India	1	1
	Bom 2/65	India	1	1
	Bom 3/65	India	1	1
	Gs 9/65	India	1	1
	H 16/64	India	1	1
	HK 130	Hong Kong	2	2
	Phil 32/62	Phillipines	2	2
V. eltor (water strains)	EW-6	Calcutta (water)	14	0*
	ME-7	Middle East	20	0

* One ligated loop showed slight swelling

Table 2. *Pathogenicity to infant rabbits*

Type of vibrio	Strain no.	Area of isolation	Total no. of tests	No. of tests showing positive reactions
V. cholerae	H 86/62	Calcutta	1	1
	H 96/64	Calcutta	2	2
V. eltor	Baroda 6/64	India	1	1
	Baroda 7/64	India	1	1
	Bg 79/64	India	1	1
	GS 9/65	India	5	5
V. eltor (water strains)	ME-7	Middle East	19	0*
	EW-6	Calcutta (water)	4	0

* One rabbit showed slight distension of the large gut with fluid.

Tests for protection by the vaccine in rabbits

It was found that a single dose of live vaccine administered intra-intestinally in adult rabbits could protect the animals against gut-inflammatory reaction on subsequent challenge with *V. cholerae* and *V. eltor* (case) strains in ligated intestinal loops. Out of 16 rabbits thus immunized, all were found to resist gut-inflammatory

reaction when challenged with a *V. eltor* (case) strain. On challenge with *V. cholerae*, 12 out of 16 rabbits showed complete immunity, 3 showed partial immunity and in 1 no immunity was found.

Out of 20 infant rabbits immunized orally with live vaccine and subsequently challenged with an El Tor (case) strain, all but 3 died showing typical distension of the large intestine.

Table 3. *Multiplication of* Vibrio cholerae *and* V. eltor *(case and 'vaccine' strains) in the gut of normal adult and infant rabbits*

Experimental model	Rabbit no.	Strain no.	Total no. of vibrios injected	Total no. recovered
Intestinal loop of adult rabbit	1	ME-7	$2{\cdot}6\times10^{7}$	$1{\cdot}8\times10^{9}$
		GS 1/65	$1{\cdot}3\times10^{7}$	$4{\cdot}0\times10^{8}$
	2	ME-7	$3{\cdot}6\times10^{5}$	$4{\cdot}5\times10^{9}$
		GS 1/65	$3{\cdot}9\times10^{3}$	$2{\cdot}8\times10^{8}$
	3	ME-7	$1{\cdot}3\times10^{4}$	$2{\cdot}0\times10^{8}$
		GS 1/65	$2{\cdot}2\times10^{4}$	$3{\cdot}1\times10^{7}$
		GS 9/65	$2{\cdot}0\times10^{4}$	$5{\cdot}6\times10^{7}$
	4	ME-7	$5{\cdot}2\times10^{4}$	$2{\cdot}7\times10^{9}$
		GS 9/65	$9{\cdot}8\times10^{3}$	$4{\cdot}2\times10^{8}$
	5	ME-7	$1{\cdot}5\times10^{4}$	$1{\cdot}8\times10^{12}$
		GS 1/65	$5{\cdot}0\times10^{4}$	$1{\cdot}0\times10^{6}$
		GS 9/65	$4{\cdot}0\times10^{4}$	$5{\cdot}0\times10^{10}$
	6	ME-7	$1{\cdot}5\times10^{4}$	$3{\cdot}6\times10^{11}$
		GS 1/65	$5{\cdot}0\times10^{4}$	$1{\cdot}0\times10^{6}$
		GS 9/65	$4{\cdot}0\times10^{4}$	$1{\cdot}9\times10^{10}$
Infant rabbit	1	GS 9/65	$2{\cdot}5\times10^{4}$	$1{\cdot}8\times10^{9}$
	2	ME-7	$1{\cdot}3\times10^{6}$	$1{\cdot}8\times10^{8}$
	3	ME-7	$1{\cdot}3\times10^{5}$	$8{\cdot}0\times10^{7}$
	4	ME-7	$5{\cdot}0\times10^{4}$	$6{\cdot}9\times10^{7}$
	5	ME-7	$5{\cdot}0\times10^{4}$	$1{\cdot}0\times10^{8}$
	6	GS 9/65	$7{\cdot}0\times10^{4}$	$3{\cdot}3\times10^{9}$
	7	GS 9/65	$7{\cdot}0\times10^{4}$	$6{\cdot}1\times10^{9}$
	8	GS 9/65	$3{\cdot}6\times10^{5}$	$3{\cdot}5\times10^{7}$

Stability of the avirulent character of the vaccine strains on animal passage

Neither of the proposed vaccine strains showed enhancement of pathogenicity after ten serial passages in the ligated loops of small intestine of adult rabbits. Identical results were also obtained when the vibrio strains were propagated ten times serially in infant rabbits by the intestinal route.

Multiplication of the vaccine strains in rabbits

From the results given in Table 3 it may be seen that in the ligated loop of intestine of adult rabbits ME-7 multiplied more rapidly than GS 1/65, a *V. cholerae* strain, and to about the same extent as GS 9/65, an El Tor (case) strain. It may also be seen from the same Table that ME-7 definitely multiplied in the infant rabbit's intestine also, although the growth rate was lower than that of GS 9/65.

Tests on multiplication of the vibrios in gut of adult rabbits after intra-intestinal immunization

From the results given in Table 4 it may be seen that the vibrios multiplied in the ligated intestinal loops of the immunized rabbits but the multiplication was much slower than in normal rabbits. It is also of interest that although the virulent strains multiplied in immunized rabbits there was no gut-inflammatory reaction in the loops even after 24 hr., whereas reaction was invariably produced in normal rabbits.

Table 4. *Comparative rates of multiplication of vibrios in intestinal loops of normal and immunized* rabbits*

Experiment	Strain no.	Inoculum (0 hr.)	Viable count at 24 hr. Normal†	Viable count at 24 hr. Immunized‡
1	2	3	4	5
1	GS 9/65	$4\cdot5\times10^{2}$	$1\cdot1\times10^{11}$	$6\cdot0\times10^{8}$
	ME-7	$5\cdot0\times10^{2}$	$3\cdot1\times10^{9}$	$4\cdot7\times10^{6}$
	569B	$3\cdot5\times10^{2}$	$9\cdot0\times10^{7}$	$2\cdot7\times10^{7}$
2	GS 9/65	$4\cdot5\times10^{3}$	$6\cdot3\times10^{9}$	$2\cdot4\times10^{7}$
	ME-7	$5\cdot0\times10^{3}$	$3\cdot8\times10^{9}$	$1\cdot3\times10^{7}$
	569B	$3\cdot5\times10^{3}$	$8\cdot3\times10^{7}$	$9\cdot5\times10^{5}$
3	GS 9/65	$4\cdot5\times10^{4}$	$6\cdot0\times10^{9}$	$4\cdot4\times10^{7}$
	ME-7	$5\cdot0\times10^{4}$	$4\cdot3\times10^{8}$	$3\cdot4\times10^{7}$
	569B	$3\cdot5\times10^{4}$	$4\cdot5\times10^{8}$	$5\cdot1\times10^{4}$
4	GS 9/65	$3\cdot4\times10^{4}$	$9\cdot1\times10^{9}$	$1\cdot2\times10^{9}$
	ME-7	$5\cdot6\times10^{4}$	$1\cdot5\times10^{10}$	$8\cdot3\times10^{8}$
	569B	$8\cdot0\times10^{4}$	$1\cdot3\times10^{10}$	$2\cdot8\times10^{9}$
5	GS 9/65	$2\cdot8\times10^{4}$	$3\cdot9\times10^{9}$	$7\cdot0\times10^{7}$
	ME-7	$1\cdot0\times10^{5}$	$7\cdot8\times10^{9}$	$2\cdot0\times10^{6}$
	GS 1/65	$1\cdot6\times10^{5}$	$1\cdot3\times10^{8}$	$5\cdot0\times10^{5}$
6	GS 9/65	$1\cdot3\times10^{6}$	$2\cdot3\times10^{8}$	$1\cdot2\times10^{7}$
	ME-7	$2\cdot6\times10^{6}$	$3\cdot3\times10^{9}$	$5\cdot9\times10^{7}$
	569B	$1\cdot5\times10^{6}$	$6\cdot0\times10^{9}$	$3\cdot6\times10^{7}$
7	GS 9/65	$1\cdot4\times10^{4}$	—	$6\cdot0\times10^{6}$
	ME-7	$7\cdot3\times10^{4}$	—	$1\cdot2\times10^{7}$
	569B	$9\cdot0\times10^{4}$	—	$1\cdot3\times10^{6}$

* Immunization was carried out by intra-intestinal injection of viable ME-7 cells 8–9 days before challenge as described under Materials and Methods.

† There was reaction in all the loops injected with GS 9/65, GS 1/65 and 569B.

‡ There was no reaction in any loop.

From the data on the rate of growth of the vibrios in intestinal loops of normal and immunized rabbits presented in Table 5 and plotted in Figs. 1–3 it is evident that in the normal rabbit the *V. eltor* (case) strain multiplied about 1000 times in 8 hr. and thereafter the viable count remained stationary up to 24 hr., whereas in the immunized rabbit loop there was only a threefold multiplication in 8 hr. and about 50-fold multiplication in 24 hr. (Fig. 1). The *V. cholerae* strain multiplied about 100-fold in 8 hr. and 1000-fold in 24 hr. in normal rabbit loops, whereas in

the loops of immunized rabbits the count showed a fivefold increase in 8 hr. and a 25-fold increase in 24 hr. (Fig. 2). In the normal loop the *V. eltor* vaccine strain multiplied 300-fold in 8 hr. and 1000-fold in 24 hr. whereas in immunized rabbits there was practically no increase in viable count even at 24 hr (Fig. 3).

All the loops of the normal animal injected with *V. cholerae* and El Tor pathogenic strain showed the typical gut-inflammatory reactions from 8 hr. onwards but in no case was there any reaction in the intestinal loops of immunized rabbits.

Table 5. *Comparative rate of multiplication of vibrios in intestinal loops of normal and immunized* rabbits*

Experiment no.	Strain no.	Viable count at: 0 hr.	4 hr. Normal‡	4 hr. Immunized‡	8 hr. Normal†	8 hr. Immunized‡	24 hr. Normal†	24 hr. Immunized‡
A	GS 9/65	$2\cdot0\times10^{6}$	$3\cdot5\times10^{8}$	$6\cdot0\times10^{6}$	$1\cdot9\times10^{9}$	$3\cdot6\times10^{7}$	$4\cdot9\times10^{9}$	$7\cdot6\times10^{8}$
	569B	$1\cdot3\times10^{6}$	$7\cdot0\times10^{7}$	$9\cdot5\times10^{6}$	$1\cdot8\times10^{7}$	$1\cdot5\times10^{6}$	$3\cdot2\times10^{9}$	$9\cdot5\times10^{7}$
	ME-7	$3\cdot0\times10^{6}$	$2\cdot3\times10^{7}$	$3\cdot5\times10^{6}$	$4\cdot3\times10^{7}$	$1\cdot6\times10^{7}$	$1\cdot1\times10^{9}$	$5\cdot5\times10^{6}$
B	GS 9/65	$3\cdot5\times10^{6}$	$1\cdot0\times10^{9}$	$1\cdot2\times10^{6}$	$5\cdot5\times10^{9}$	$2\cdot2\times10^{6}$	$1\cdot2\times10^{9}$	$2\cdot7\times10^{7}$
	569B	$3\cdot5\times10^{6}$	$3\cdot9\times10^{8}$	$1\cdot0\times10^{5}$	$1\cdot5\times10^{9}$	$5\cdot8\times10^{7}$	$3\cdot6\times10^{9}$	$2\cdot5\times10^{6}$
	ME-7	$1\cdot9\times10^{6}$	$1\cdot3\times10^{8}$	$2\cdot0\times10^{7}$	$1\cdot3\times10^{10}$	$5\cdot5\times10^{4}$	$1\cdot1\times10^{10}$	$6\cdot5\times10^{5}$
C	GS 9/65	$2\cdot6\times10^{6}$	$9\cdot0\times10^{5}$	$7\cdot5\times10^{4}$	$2\cdot0\times10^{9}$	$1\cdot0\times10^{4}$	$2\cdot3\times10^{8}$	$5\cdot9\times10^{7}$
	GS 1/65	$1\cdot5\times10^{6}$	$1\cdot6\times10^{8}$	$2\cdot1\times10^{4}$	$1\cdot1\times10^{9}$	$5\cdot3\times10^{6}$	$3\cdot6\times10^{9}$	$6\cdot0\times10^{7}$
	ME-7	$2\cdot5\times10^{5}$	$2\cdot1\times10^{6}$	$4\cdot5\times10^{4}$	$2\cdot2\times10^{9}$	0	$3\cdot3\times10^{9}$	0

* Immunization was carried out by intra-intestinal injection of viable ME-7 cells 8–9 days before challenge as described under Materials and Methods.

† There was reaction in all the loops injected with GS 9/65, GS 1/65 and 569B.

‡ There was no reaction in any loop.

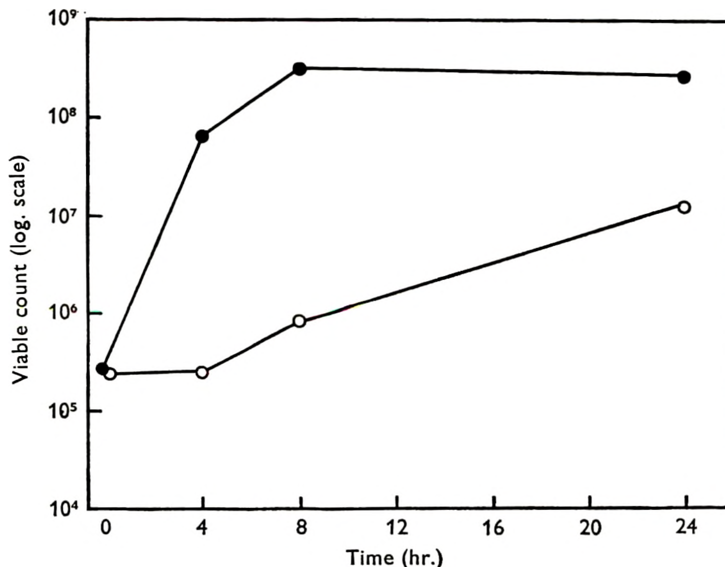

Fig. 1. Growth of pathogenic *Vibrio eltor* (GS 9/65) in the intestinal loop of normal and immunized rabbits. ●, Normal rabbit; ○, immunized rabbit.

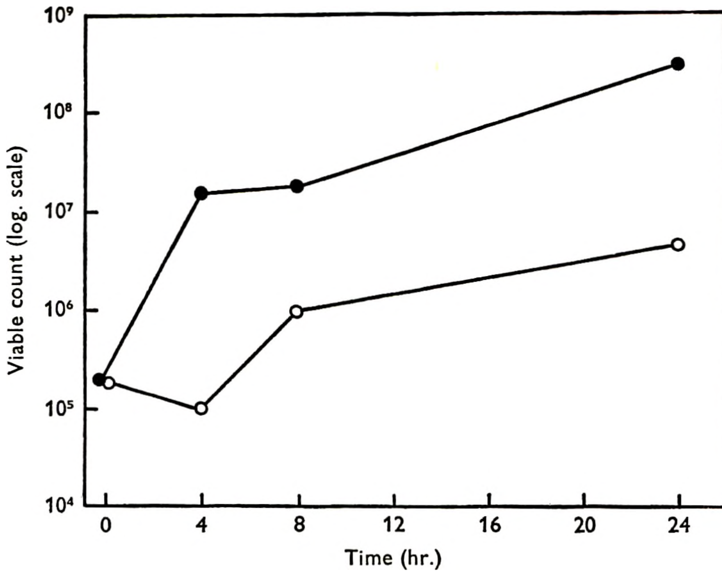

Fig. 2. Growth of *Vibrio cholerae* (569B) in the intestinal loop of normal and immunized rabbits. ●, Normal rabbit; ○, immunized rabbit.

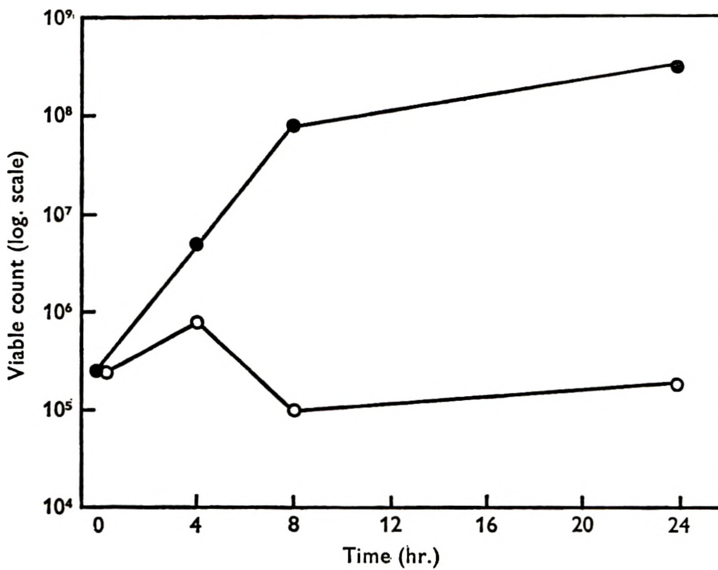

Fig. 3. Growth of the vaccine strain (ME-7) in the intestinal loop of normal and immunized rabbits. ●, Normal rabbit; ○, immunized rabbit.

DISCUSSION

It may be seen from the results in Tables 1 and 2 that the El Tor vaccine strains ME-7 and EW-6 proved apathogenic both in the ligated intestinal loops of adult rabbits and on intra-intestinal challenge in infant rabbits in marked contrast to cultures of *V. cholerae* and *V. eltor* strains isolated from cholera patients. Absence

of pathogenicity of the vaccine strains was confirmed to be a stable characteristic which remained unaffected by serial animal passages. However, the results of pathogenicity tests with the vaccine strains appears to have differed to some extent in different laboratories, which may be due to some difference in susceptibility to experimental cholera of rabbits from different stocks. The results presented here show that injection of a dose as high as $1{\cdot}0 \times 10^9$ viable cells of the vaccine strains failed to produce choleraic reaction in infant rabbits. The relative avirulence of the vaccine strains has been confirmed by Finkelstein, Norris & Dutta (1964). Dr John C. Feeley of the National Institute of Health, Bethesda, U.S.A., Dr Rolf Freter of the University of Michigan Medical School, U.S.A., and Dr H. Ogonuki of Chiba Serum Institute, Japan, to whom cultures of the water strains were sent for pathogenicity tests, also found them avirulent. J. C. Feeley (personal communication), after injecting over $1{\cdot}0 \times 10^9$ viable cells of ME-7 and EW-6 directly into the small intestines of infant rabbits or administering a similar number of vibrios orally, could not elicit diarrhoea, whereas diarrhoea was produced in infant rabbits given $1{\cdot}2 \times 10^2$ and $1{\cdot}5 \times 10^4$ living cells of a known virulent strain (VC 12). But he could obtain positive cultures at autopsy from all animals which had been given the water strains, except in one case of intra-intestinal challenge with ME-7; however, the latter animal was positive by rectal culture at 48 hr. after challenge, as were all other animals.

R. Freter (personal communication) after injecting $1{\cdot}0 \times 10^6$ viable cells directly into the small intestine, found that all animals receiving EW-6 and ME-7 survived the 20 hr. experimental period. There was a small amount of fluid accumulating in all the three animals receiving EW-6 and in one of the three receiving ME-7, but the quantity of fluid found was much less than with the two virulent strains he used.

H. Ogonuki (personal communication) also found ME-7 and EW-6 strains in heavy dose to be apathogenic in the infant rabbit test.

The finding of an occasional mild reaction produced by the vaccine strains is in accord with Mukerjee's observation (Mukerjee, 1963). Oza and Dutta's observation (Oza & Dutta, 1965) also indicates that these strains are not completely devoid of pathogenicity in experimental animals as the virulence of these strains could be enhanced by adding mucin, and EW-6 was found to be choleragenic even without addition of mucin. They used an unusually heavy inoculum (10^9 vibrios/100 g. of body weight of infant rabbit).

The residual virulence, which the water strains appear to possess, is an essential requirement for a live vaccine strain intended to stimulate the immunologically competent cells at the site of multiplication (Emel'janova, 1957). A completely avirulent strain is likely to be eliminated as an inert substance from the intestinal tract without causing any immunogenic reaction in the intestinal mucous membrane.

The vaccine strains administered by the intra-intestinal route have been found to protect adult rabbits against gut inflammatory reaction on subsequent challenge in ligated intestinal loops with both *V. cholerae* and *V. eltor* (case) strains. Similar protective immunity was not produced by oral vaccination of infant rabbits. The

failure of the vaccine to protect the infant rabbits was presumably due to their immaturity; it is known that the immunity-forming mechanism is not well developed in immature animals (Good & Papermaster, 1964).

Freter in recent years has done considerable work on the possibility of developing an oral cholera vaccine, but he used killed vaccines in his studies. Freter (1965) agreed that a live oral cholera vaccine should be better than killed cultures provided that the vaccine strain is capable of multiplication in the human intestinal tract. He felt that the low virulence of the vaccine strains proposed by Mukerjee was an expression of their inability to multiply in the lumen of the intestine. However, from the results reported in this paper it is clear that the vaccine strains are capable of multiplying in the gut of adult and infant rabbits. Dr Feeley observed that the strains survived in the intestine of infant rabbits at least up to 5 days (the period of his observation). The multiplication of the strains without giving rise to pathological reactions indicates that they are incapable of producing choleragenic toxin in the intestinal tract in sufficient concentration to give rise to an observable reaction in the animal model.

It is clear from the results presented here that multiplication of all the vibrio strains was markedly slower in immunized rabbits' gut than in the normal rabbits' gut. The difference was most marked during the first 4–8 hr., when in normal rabbits all the vibrio strains multiplied rapidly while in the immunized rabbits the multiplication was very sluggish. The total viable count of the vibrios in the intestinal loops of immunized rabbits at the end of 24 hr. also was less than in the loops of normal rabbits. The rates of growth of the vaccine strain, pathogenic *V. eltor* and *V. cholerae* in the immunized rabbits' loop increased in that order.

The inhibition of vibrio growth in immunized rabbits' intestinal loops may be due either to the soluble antibodies present in the lumen or to the phagocytic activity of the reticulo-endothelial cells of the intestine, the phagocytic index of which might have been increased by the previous exposure to antigen. However, the interval during which growth is slowed down was found to be constant (4 hr.) irrespective of the vibrio count in the inoculum, whereas if the initial phase of inhibition had been due to either specific antibodies or phagocytosis slowing down growth till such time as the antibacterial or phagocytic mechanism became overwhelmed, the period of growth-inhibition should have varied according to the size of the challenge dose of the vibrios.

The slower rate of vibrio multiplication is not likely to be the principal reason for the absence of gut-inflammatory reaction in immunized rabbits on challenge with virulent strains, since a total viable count of $1{\cdot}0 \times 10^8$ cells of pathogenic vibrios invariably produced reactions in intestinal loops of the normal rabbit, whereas in immunized rabbits' loops the same strain of vibrio multiplied to counts as high as $2{\cdot}8 \times 10^9$ cells in 24 hr. and yet failed to cause any reaction. This result suggests that apart from antibacterial antibodies, some degree of antitoxic immunity was produced as a result of multiplication of the vaccine strain in the intestine of vaccinated animals. Although it has not been possible to demonstrate production of soluble toxin by the vaccine strains in *in vitro* culture (R. A. Finkelstein and J. C. Feeley, personal communication), this does not constitute

unequivocal evidence that the vaccine strains are incapable of producing toxin in subminimal concentrations under suitable conditions. The failure to demonstrate choleragenic toxin in culture filtrate may equally well be explained on the supposition that the toxin concentration was insufficient to cause choleragenic symptoms in rabbit models, and a more sensitive method of assay might have enabled its detection. An alternative explanation of the absence of choleraic reaction in the intestinal loops of immunized rabbits is that the metabolism of the pathogenic vibrios may have been altered in such a way that they do not get the proper nutrient conditions required for the production of choleragenic toxin. An alteration in metabolic capacities is not unlikely in view of the observed effect on the dynamics of growth, and the work of Finkelstein *et al.* (1964) has established that even a prolifically toxinogenic strain forms toxin only when specific nutrient requirements are available in the culture medium.

The vaccine strains thus appear to be capable of getting established in the intestine and of multiplying there without causing disease. As a result of their multiplication, there is production of protective immunity in the intestine, which comprises both antibacterial and antitoxic activities. The naturally avirulent El Tor strains selected for study therefore appear to fulfil all the requirements of a live vaccine strain, as judged from the present laboratory tests.

SUMMARY

The possibility of developing a living oral cholera vaccine with naturally avirulent El Tor cultures isolated in a Middle East country or from water sources in Calcutta in the absence of cholera El Tor in these areas has been further studied.

In an extended series of experiments the markedly low pathogenicity of the proposed vaccine strains in laboratory animals has been confirmed. The vaccine strains have been shown to get established and multiply regularly in the ligated intestinal loops of adult rabbits and in the intestine of infant rabbits without producing pathogenic reactions.

The apathogenic character of the vaccine strains has been found to be stable. When propagated serially in these two laboratory models of experimental cholera the vaccine strains show no enhancement of pathogenicity.

Intra-intestinal administration of the live vaccine has been shown to protect adult rabbits fully in the intestinal loop test. Immunized animals were also protected against challenge with *V. cholerae* strains, though to a somewhat lesser extent. Protective immunity could not, however, be demonstrated in infant rabbits probably because of the immunity-forming mechanism being still rudimentary.

Immunization with live vaccine was found to inhibit the growth of homologous strains in the ligated intestinal loop of the adult rabbit. The growth rate of pathogenic *V. eltor* and *V. cholerae* strains was also seen to be markedly reduced throughout the 24 hr. period of observation, but more markedly during the first 4–8 hr. However, the total count of pathogenic *V. eltor* and *V. cholerae* strains at 24 hr. after inoculation in ligated ileal loops of immunized rabbits reached levels that invariably caused inflammation and accumulation of fluid in normal rabbits'

loops. The absence of gut-inflammatory reaction in immunized rabbits under these conditions has been discussed in relation to the nature of the immunity produced by administration of live vaccine.

We are grateful to Dr R. A. Finkelstein of SEATO Medical Research Laboratory, Bangkok, Dr John C. Feeley, National Institutes of Health, Bethesda, U.S.A. and Dr H. Ogonuki, Chiba Serum Institute, Chiba, Japan, for their help in testing the vaccine strains for their apathogenic character.

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