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Contaminant viruses in two live virus vaccines produced in chick cells

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(*Received* 27 *May* 1965)

Attention has been focused on the need for particular care in the preparation of virus vaccines since the discovery that a virus (SV 40) present as a contaminant in Salk-type poliomyelitis vaccines could produce tumours following inoculation into rats and hamsters (Eddy, Borman, Berkeley & Young, 1961; Girardi, Sweet, Slotnick & Hilleman, 1962) and could, moreover, escape the inactivation procedures used in the preparation of such vaccines.

Our attention has been directed towards live virus vaccines containing virus grown in eggs or in chick embryo cell cultures. Fowls are known to harbour leukosis viruses which are transmitted from hen to egg so that the embryos are carriers of the virus. The use of the eggs for the growth of viruses such as 17D strain yellow-fever, or attenuated measles, might result in a considerable contamination of the vaccine with leukosis-group viruses. Very few flocks of chicken are known to be completely free from leukosis and, at the time these tests were made, the yellow-fever and measles viruses were *not* being propagated in tissue from such flocks.

Two experimental approaches were adopted. The first was to neutralize the yellow-fever virus with a large excess of monkey antiserum (from a monkey inoculated with a *non-avian* strain of yellow-fever virus) and the measles virus with rabbit antiserum and to assay the residual material for leukosis virus using the interference test devised by Rubin (1960). The second was to immunize adult chickens of a flock maintained in isolation with the neutralized viruses and test the sera for capacity to neutralize Rous sarcoma virus of the Bryan strain (RSV(B)) by the general procedure of Simons & Dougherty (1963).

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MATERIALS

Preparation of the vaccines

The two vaccines studied were prepared by different methods. Both are live-virus vaccines and use the seed-lot systems (Fox, Kossobudzki & da Cunha, 1943). In the case of yellow-fever vaccine (YFS 2) the virus was grown by inoculating seed virus into 7-day-old chick embryos with continued incubation in the intact egg for a further 4 days; the infected embryos were then harvested, homogenized in saline and the clarified embryonic pulp freeze-dried in batches of convenient size. The sample tested was taken before freeze-drying from the seed lot used for routine production between 1958 and 1964 and was composed of a pool of approximately 2000 embryos derived from seven commercial flocks in southern England. It had been stored in sealed glass containers at $-70°$ C. until immediately before test. The YF virus content was $10^{5·8}$ mouse LD 50 per ml. and was only one chick embryo passage from original Rockefeller Institute virus prepared by Dr Max Theiler in 1944.

The experimental vaccine (MV 16) was prepared from the fluid medium of chick embryo cell cultures infected with an attenuated measles virus (Goffe & Laurence, 1961). The virus strain was derived from Enders' Edmonston 'B' strain and was in its third tissue culture passage after a series of thirty serial chick-embryo passages by the intra-amniotic route. The tissue cultures were prepared by trypsinizing decapitated 8-day chick embryos and growing the cells in a monolayer. This batch was grown from a pool of thirty-five embryos from a single flock. Virus was added on the third day, and incubation continued in a serum-free medium for a further 8 days. The medium containing the virus and some degenerated cells was harvested and clarified by centrifugation; the supernatant fluid together with a stabilizer was freeze-dried to form the final vaccine. The sample tested was in the final freeze-dried form exactly as used for the inoculation of children (Benson *et al.* 1964; Watson, 1965).

METHODS AND RESULTS

Interference test for leukosis virus in yellow fever vaccine YFS 2

A sample of yellow fever vaccine YFS 2 was neutralized with monkey antiserum by incubation of equal volumes of virus and antiserum (diluted 1/10) at 4° C. for 24 hr. Secondary chick embryo fibroblast cultures were set up from leukosis-free embryos and infected in suspension with 0·1 ml. of the undiluted vaccine-serum mixture or a 1/10 dilution according to Rubin (1960). These cultures and uninfected controls were then passaged and challenged with RSV (B) 5 days later. The results (Table 1) show that the relative sensitivity to RSV (B) of the infected cultures was much reduced compared with the controls. This indicates that the neutralized yellow fever vaccine contains very significant amounts of a virus which, in this test, acted like a member of the fowl leukosis group.

Two similar tests for interference with RSV (B) were made with measles vaccine, using Measles Vaccine 16 Unspun, 11,15.3.62 (25 ml.) neutralized with Measles

Rabbit Immune Serum Pool no. R. 10 (3 ml.) The results are shown in Table 2. In these tests four tissue culture passages of the infected cells were required before their relative sensitivity to RSV(B) approximated to that produced by only one passage of the cells infected by the yellow fever vaccine-serum mixture (Table 1). Since the purpose of the passages is to increase the titre of the interfering virus, this means that the measles vaccine contained less interfering virus than the yellow-fever vaccine.

Table 1. *Interference test on yellow-fever vaccine*

(A relative sensitivity of 0·001 means that the cultures previously infected with the interfering virus required 10^5 focus-forming units (F.F.U.) of RSV(B) to produce the same number of foci as 10^2 F.F.U. in the controls (or 10^3:1).)

Infected with	Tissue culture passage no. 1
YFS 2 vaccine-serum mixture	0·001*
YFS 2 vaccine-serum mixture diluted 1:10	0·002

* Relative sensitivity to challenge with (RSV(B).

Table 2. *Interference test on measles vaccine*

	Tissue culture passage no.			
Infected with	1	2	3	4
MV 16 virus-serum mixture	0·5*	0·02	0·02	—
MV 16 virus-serum mixture	—	0·1	—	0·008

* Relative sensitivity to challenge with RSV(B).

Both yellow-fever and measles virus are cytopathic for chick embryo cells in culture. If the viruses are inadequately neutralized lytic plaques appear. For this reason antiserum was kept in all the cultures at each passage. Under these conditions, which were based on model experiments, no 'break-through' of the neutralization could be detected. It would appear, therefore, that the observed interference cannot be attributed to residual yellow fever or measles virus. Moreover, interference of this type, increasing in extent with the number of subcultures of the cells, has been shown by Hanafusa, Hanafusa & Rubin (1964) to be dependent upon antigenic similarity between the interfering virus and the challenge RSV(B) stock. A virus with these properties is most likely to be a virus of the avian leukosis group.

Immunity tests

Equal numbers of adult male and female Edinburgh Brown Leghorns were randomly allotted to four groups. Each group, consisting of fourteen or fifteen birds, was housed in isolated arks kept in the open on a concrete apron. Food and water were provided *ad libitum*.

Sera from all birds were collected 24–31 days before the primary inoculation, which consisted of the following preparations:

Group 1: Freund's adjuvant plus undiluted neutralized yellow fever vaccine.

Group 2: Freund's adjuvant plus undiluted neutralized measles vaccine.

Group 3: Freund's adjuvant plus dilute lymphoid leukosis virus.

Group 4: Freund's adjuvant plus concentrated lymphoid leukosis virus.

Each bird received 2·0 ml. of the relevant preparation, 0·5 ml. being given intramuscularly at each of four sites in the pectoral muscles. Twenty-six days later sera were collected. On the same day the birds were given the following treatment as a second inoculum:

Group 1: One ml. of neutralized yellow fever vaccine comprising 0·5 ml. subcutaneously into each wing web.

Group 2: 0·5 ml. of neutralized measles vaccine subcutaneously into the right wing web.

Group 3: One ml. of dilute lymphoid leukosis virus, 0·5 ml. subcutaneously into each wing web.

Group 4: One ml. of concentrated lymphoid leukosis virus, 0·5 ml. subcutaneously into each wing web.

Seven days later sera were again collected.

Birds of groups 1, 2 and 3 were killed 6 months later and the birds of group 1 examined for the presence of tumours. No tumours were found.

The lymphoid leukosis virus used was HPRS F42, which has been shown to interfere with the multiplication of RSV(B) virus *in vitro* and to reproduce lymphoid leukosis following inoculation of day-old chicks (Biggs & Payne, 1965).

For the neutralization tests on these sera the virus used was a Moloney-type preparation—a single batch of RSV(B) stored in small volumes at $-70°$ C. in sealed ampoules. The infectivity of this preparation for the chorioallantoic membrane of eggs of the Brown Leghorn strain was such that an inoculum of 0·1 ml. of a dilution of $1{\cdot}5 \times 10^{-4}$ gave about 100 pocks. Before admixture with the virus the sera were diluted 1/5 with medium DI (phosphate-buffered saline containing 2% inactivated calf serum and 100 units/ml. of penicillin and streptomycin) and inactivated at 56° C. for 30 min. Equal quantities of virus and serum were mixed, incubated overnight at 4° C. and 0·1 ml. volumes inoculated into groups of six eggs by the method of Simons & Dougherty (1963). The infected eggs were incubated at 38° C. for a further 7 days, chilled at 0° C. overnight, the chorioallantoic membranes removed, and the pocks counted.

The results may conveniently be expressed as percentage reduction of pock count as between untreated virus and virus incubated with pre-immunization or primary or secondary challenge sera. Evidence that the serum has significant virus neutralizing capacity will be accepted if the pock count in the challenge group is 15% or less of that of the control figures. Table 3 sets out these data for birds of group 1.

Since the birds in groups 2, 3 and 4 showed less indication than this of having been immunized, the results for these groups are only summarized in Table 4.

All groups of chickens were treated with preparations which had been shown by the RSV(B)-interference test to contain an avian leukosis virus. However, chickens treated with measles vaccine (group 2) and dilute and concentrated preparations

of HPRS F42 (groups 3 and 4) did not produce antibody to RSV(B) during the period of this experiment.

Failure to elicit antibodies with concentrated leukosis virus (HPRS F42) under these conditions may be a result of antigenic differences between F42 and RSV(B), or of failure to achieve adequate immunization of the fowls. Accordingly, nine birds of group 4 were re-inoculated 180 days later with HPRS F42 and bled 21 days afterwards. Eight of the nine sera had significant neutralizing capacity for RSV(B).

Table 3. *Neutralizing capacity for RSV(B) of sera from chickens in group* 1

Bird	% Control pock count			Inhibitory
	Pre-bleed	Primary	Secondary	
Female 1	70	31	40	
2	38	40	38	
3	38	24	25	
4	69	27	15	3/7
5	73	60	47	
6	100	37	15	
7	77	27	15	
Male 1	32	37	—	
2	84	3	1	
3	32	45	41	
4	68	10	14	
5	87	6	0	6/7
6	90	41	15	
7	41	8	1	
8	87	65	3	

Table 4. *Neutralizing capacity for RSV(B) of sera from chickens in groups* 2–4

	Sex	Inhibitory
Group 2	Female	0/7
	Male	0/8
Group 3	Female	0/6
	Male	0/8
Group 4	Female	1/6
	Male	0/6

Another group of ten birds was subjected to a different immunization procedure with the same virus, as follows: day 1, inoculated with 2×10^4 focus-forming units (FFU) of HPRS F42 intravenously; day 7, and at 7-day intervals for five weeks, given 5×10^4 FFU of virus intramuscularly. On the 14th day one bird showed antibodies to RSV(B). On day 21 four birds, and on day 56 all 10 birds had antibodies to RSV(B).

Two different leukosis virus strains (HPRS F45 and HPRS B15) administered to the same schedule gave respectively 4 out of 9 and 5 out of 8 neutralizing sera on the 51st and 48th days respectively.

With the collaboration of a number of R.A.F. men who consented to being bled before and after standard vaccination against yellow fever, it was possible to test

for antibodies to avian leukosis virus in man using the same time relationships as for group 1 birds but without re-immunization. None of the twelve volunteers developed any antibodies to RSV(B) in his serum as a result of his vaccination.

DISCUSSION

We conclude from this investigation that these samples of yellow fever and measles vaccines each contained a virus, presumably acquired from infected chick embryo tissues, with the biological properties of an avian leukosis virus. Adult male volunteers vaccinated with the yellow-fever vaccine did not develop detectable antibodies to this contaminant virus. Some strains of Rous sarcoma virus, which is a member of this chicken tumour virus group, will infect human and simian tissues *in vitro* (Jensen, Girardi, Gilden & Koprowski 1964), produce chromosome abnormalities in human leukocytes (Nichols *et al.* 1964) and even tumours in monkeys (Munro & Windle, 1963).

The regulations for the manufacture of live measles vaccine both in Britain and the U.S.A. now require that the fertile eggs for chick embryo cell cultures shall be derived from leukosis-free flocks and that the final vaccine shall be tested for the presence of leukosis virus. Our results show that the interference test is a much more sensitive indicator of the presence of leukosis virus than the immunity test, but both require embryos or birds from leukosis-free flocks.

There is no evidence that the contaminant virus in yellow-fever vaccine is dangerous—it will not be present in measles vaccine—but, nevertheless, it would now be prudent to adopt similar precautions for its manufacture to those in force for measles vaccine. At the same time it would be worth while looking for any positive (or negative) association between human malignancy, especially leukaemia, and prior yellow-fever vaccination, especially as some 20 years have now elapsed since the introduction of this vaccine.

SUMMARY

Samples of yellow-fever vaccine prepared from homogenized chick embryos, and of an experimental measles vaccine prepared from chick embryo cells, have each been shown to contain a contaminant virus similar in properties to an avian leukosis virus. Young adult males injected with the yellow-fever vaccine did not develop neutralizing antibodies for Rous sarcoma virus.

We should like to thank Prof. A. J. Haddow of the East African Virus Research Institute, Entebbe, for supplying us with the monkey yellow-fever antiserum. T. C. Hirst and Mrs K. A. Denny gave us invaluable assistance.

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The microbiological and epidemiological properties of infections caused by *Salmonella enteritidis*

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In recent years in many countries, in contrast to the decreasing incidence of typhoid and paratyphoid fevers, there has been a considerable rise in the number of infections caused by other types of *Salmonella* (Brodhage, 1963; Mihalyfi, Kende, Jonas & Vamos, 1961; Galton, Steel & Newell, 1964; Peluffo, 1964; Seeliger & Maya, 1964; Silberstein & Gerichter, 1964; Stahn, 1963). In Poland in 1957 infections with salmonellas, excluding *S. typhi* and *S. paratyphi A* and *B*, were found in 994 sick and 780 healthy persons, and the corresponding figures for 1963 are 3047 and 2369 respectively (Buczowski, unpublished data). In 1964 the number of cases of salmonellosis increased still more. Though in previous years the dominant type was *S. typhimurium* (65–75 % of the total) from 1963 *S. enteritidis* infections were the most frequent. Most of these infections were in infants, and occurred in the form of more or less intensive hospital epidemics.

Since there have been very few reports of infections with *S. enteritidis* (Crainz, Lombardo & Romagnoli, 1960; Kanski, Moszkowska & Skunska, 1947; Nowgorodzkaja, 1957; Swicowa, Kulczynska & Prus, 1962) it appears useful to present observations on certain microbiological and epidemiological properties of these infections.

DESCRIPTION OF THE EPIDEMIC

Up to 1964, *S. enteritidis* was very rarely found in the area investigated. Of a total of more than 3300 cultures of *Salmonella* isolated in the years 1957–63, only three were identified as *S. enteritidis*. The first cases in the epidemic were diagnosed in June 1964, and up to February 1965 there was a total of 309 cases registered* (Fig. 1). In reality the epidemic had probably begun 3–4 months earlier, but unfortunately the disease at this time was treated as a 'virus infection'. Of the total number of cases observed, 293 were discovered among children treated for various reasons in six hospital wards, the remainder were in out-patients or among healthy persons (Table 1). The infection rate with *S. enteritidis* varied from 12 to 70 % of the patients in the affected wards. The hygienic conditions varied from ward to ward, but no strict correlation could be found between these conditions

* At the time when this paper was submitted for publication the number of registered cases had increased to 494.

and the incidence of infection. It was established that the epidemic began in the neonatal ward in the hospital at Lukow, and spread thence by infected children to the infectious and children's wards of the same hospital, and from there to the children's wards of hospitals in Lublin and Radzyn.† The age distribution of cases was from 2 days to 65 years, almost half were children under 6 months (Table 2).

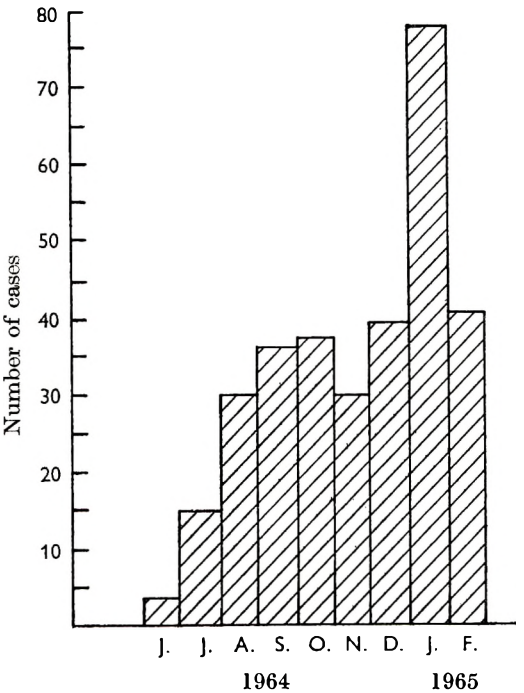

Fig. 1. The epidemic of Salmonellosis in the Lublin province (June 1964–February 1965).

Among hospital personnel eleven cases were noted, but investigations among them were not carried out regularly. It was noted that as the epidemic continued and spread, the number of sporadic cases, not connected with hospital foci, also grew. It is at present difficult to say whether the infection is getting the characteristics of endemicity.

In clinical investigations it was possible to differentiate four forms: gastro-enteritis, 57 % of the total; paratyphoid-like, 5 %; cases with predominant symptoms in the upper respiratory tract, 31 %; inapparent infections, 7 %. In the young the disease was usually severe, the death-rate in the newborn being as high as 25 %, in infants 6–8 %. In older patients the infection usually took the form of slight enteric disorders, or inapparent infection.

† More detailed data concerning these foci are presented in our paper to be published in *Revue Hyg. Méd. soc.*

Table 1. *Cases of* Salmonella enteritidis *infection detected among patients in particular hospital wards, and other persons*

(1) Hospital of the Lukow region		
Neonatal department	42	
Infants' department	83	
Infectious-diseases department	59	
Total		184
(2) Children's Hospital of the Lublin Medical Academy		
Infants' department no. 5	57	
Infants' department no. 4	8	
Other wards	2	
Total		67
(3) Hospital of the Radzyn region		
Children's department		42
(4) Sporadic cases not directly connected with hospital foci		16
Total cases		309

MATERIALS AND METHODS

A total of 309 cases of infection were examined microbiologically. Altogether 4075 samples were tested, of which 2003 were samples of faeces from 303 persons, 1024 were samples of urine from 203 persons, 328 were blood samples from 145 persons, 442 were swabs from the upper respiratory tract in 131 persons and 278 were blood samples from 139 persons, for serological examination. The investigations of faeces, urine, blood and swabs from the respiratory tract were made according to the methods generally accepted in Poland, using liquid selective-enrichment media. Sera were tested for agglutinins against *S. typhi*, *S. paratyphi A* and *B* and organisms containing gm antigen. In the wards affected by the epidemic, twenty-five air-samples were also taken, by means of bacteriological filters in a 'Staplex' type respirator. Aspiration of the air was made at a height of 1·5 m. and at a distance of 1–3 m. from the beds of the infected babies. In every case we carried out an epidemiological interview to attempt to discover the source and mode of infection, and 346 members of patients' families had their faeces examined 1–3 times.

RESULTS

From samples of blood, faeces, urine and mucus from the upper respiratory tract 1608 cultures of *S. enteritidis* were obtained. The distribution of these positive cultures is shown in Table 3, where it is seen that only 7 out of 303 patients did not show at least one positive culture from the faeces. More than 70% were persistent faecal excreters, and the remainder were intermittent excreters with negative periods of from 10 to 20 days. More than 50% of the positive faecal cultures were from normal stools, but the frequency of isolation of salmonellas from loose stools containing mucus or blood was significantly higher than that from normal stools.

The number of urinary excretors was very high—138 out of 203 investigated

Table 2. *Distribution of* Salmonella enteritidis *cases according to sex and age*

	Age										
	2–14 days	15–30 days	31–60 days	61–90 days	4–6 months	7–12 months	1–3 years	4–6 years	7–14 years	Over 14 years	Total
Male	11	7	15	15	30	26	21	10	22	8	165
Female	9	5	19	19	23	11	23	8	13	14	144
Total	20	12	34	34	53	37	44	18	35	22*	309
%	10·4		22·0		17·1	12·0	14·2	17·1		7·2	100·0

* Eleven were hospital staff.

Table 3. *Results of bacteriological examination of* 309 Salmonella enteritidis *patients*

Investigated materials	Number of patients			Number of positive findings		Distribution of patients according to number of positive findings							
	Investigated	With positive findings	%	Total	Average per patient	1	2–3	4–5	6–7	8–9	10–14	15–19	20–39
Faeces	303	296	97·7	1090	3·7	92	83	64	28	12	12	5	0
Urine	203	138	68·0	377	2·7	49	59	17	11	2	2	0	0
Blood	145	25	17·2	26	1·0	24	1	0	0	0	0	0	0
Respiratory tract	131	37	27·5	115	3·0	16	9	4	4	2	2	0	0
Total	309	309	100·0	1608	5·2	83	80	43	37	23	20	8	15

Of these, 63 were males and 75 females. All specimens of urine from females were collected with a catheter. Urinary excretion usually began much later in the disease than faecal excretion, sometimes as much as 15–25 days after the onset of infection. It was usually persistent, but small numbers of patients showed negative intervals of from 20 to 60 days. Of the positive urine cultures, 16 % were taken from patients with fever, in the acute stage of their illness, and 84 % during afebrile periods.

S. enteritidis was cultured from the blood of 25 infected persons, on two occasions from one of them. Of the 26 cultures, 23 grew within 24 hr. in the incubator, suggesting a relatively heavy blood infection: the other three strains grew after 3–5 days incubation. The clinical course of the disease in children with bacteraemia was usually severe, and 25 % of them died. It is noteworthy that the blood culture was positive in only 18 of 83 urinary excreters whose blood was tested, suggesting that, if the urinary tract was infected via the blood-stream, the bacteraemia was of short duration.

Table 4. *Correlation between number of respiratory tract samples examined and isolation of* Salmonella enteritidis

No. of samples investigated from one patient	No. of patients examined	No. of detected carriers	%
1–2	67	12	17·9
3 and more	64	25	39·0

$\chi^2 = 5{\cdot}656$; N = 1; $P = 0{\cdot}02$.

In the upper respiratory tract *S. enteritidis* was isolated from 37 of 131 children examined, 2 from the nose and throat and 35 from the throat alone. It is seen from Table 3 that many of these were repeatedly positive. Throat carriers were found in all the hospital wards involved in the epidemic. They were usually persistent carriers, but a few showed long negative periods followed by a positive result; this may have been due to reinfection. A close correlation was found between the frequency of detection of *S. enteritidis* in the upper respiratory tract and the number of samples examined (Table 4).

In 12 cases isolation of *S. enteritidis* occurred simultaneously with isolation of other bacteria of the *Enterobacteriacea* group: in 10 with *Shigella flexneri* and in 2 with *Salmonella typhi*. These patients had contracted salmonellosis during their treatment in the infectious-diseases ward.

From other materials investigated, 10 strains were isolated: 2 from pus from the ear, 3 from cerebro-spinal fluid and 5 from lung, heart and lymphatic gland tissues taken during autopsies.

S. enteritidis was isolated from 2 out of 25 air samples taken from wards in which respiratory carriers occurred.

Resistance of Salmonella enteritidis *to antibiotics*

All strains isolated from blood, faeces, urine, upper respiratory tract and air were highly resistant to penicillin, chloromycetin, aureomycin, terramycin, tetracyclin and neomycin. They were only weakly sensitive to streptomycin (zone of inhibition = 15–18 mm.) Resistance to antibiotics was found in strains isolated both in the acute stage of disease and in convalescents. Preliminary data also suggest that the clinical effect of these antibiotics was very slight. Antibiotics had no effect upon the carrier-state. Our observations here agree with those of other authors (Seligmann, Barash & Cohlan, 1947; Neter, 1950; Datta & Pridie, 1960).

Table 5. *Examination of blood sera with gm antigen in salmonellosis patients and control group*

Group	No. of examined persons	Agglutination test negative	Agglutination test positive—max. titre				
			1/50	1/100	1/200	1/400	1/800 or more
Salmonellosis	139	50	16	18	22	21	12
Control	127	122	3	1	0	1	0

The results of serological examinations

The agglutination test was performed with gm antigen on 139 individuals with bacteriologically confirmed salmonellosis. In order to check the specificity of this test it was simultaneously performed on 127 sera from patients with typhoid fever, dysentery and other diagnoses and from healthy blood donors. As is seen in Table 5, only two positive results are found in the control group, while in those infected with *S. enteritidis* a titre of 1/100–1/800 or more was found in 52 % of those examined. This suggests that a titre of 1/100 is specific for these infections and has diagnostic significance. The highest titres were found in the second to the third weeks after onset. In a third of those investigated the agglutinins persisted up to 3 months or more after the onset of infection, suggesting that the test might be useful in retrospective investigations. An increase of titre in the course of infection was not always observed. In patients with bacteraemia or bacteriuria up to 80 % showed positive agglutination reactions; this was 4–5 times higher than in those who were faecal excreters only. Of 22 throat carriers in whom the agglutination test was carried out, 17 had a titre of 1/100–1/1600.

It is noteworthy that there was a considerable number of positive sera from infants up to 3 months of age, in all 48 sera from 36 infants. In nine cases the sera of the mothers of these positive infants were examined, all with negative results.

The period of carrier-state and the significance of age

The frequency of positive faecal cultures and of positive serological results were independent of age (Table 6). Significant differences were, however, found as regards other kinds of examinations. Above 14 years of age, bacteria in urine were found in six samples only (two persons), cultures from blood were obtained

only in children up to the third year of life, and of 115 cultures from the upper respiratory tract, 90 were obtained from infants up to the sixth month of life, 24 from infants aged from 7 months to 3 years, and only 1 from a 6-year-old child. Throat-carriers were also not found among 73 personnel of the Children's Wards, who were examined 2–3 times.

Table 6. *Frequency of* Salmonella enteritidis *isolation from various materials in relation to age of the patients (number of samples)*

Age group (at the onset of infection)	Faeces		Urine		Blood		Respiratory tract		Agglutination test	
	Ex.*	Pos.†	Ex.	Pos.	Ex.	Pos.	Ex.	Pos.	Ex.	Pos.
2–14 d.	79	67	29	25	10	6	9	5	3	1
15–30 d.	70	38	35	17	12	1	22	6	8	1
1–3 m.	546	307	294	102	93	9	110	34	82	46
4–6 m.	489	252	282	105	89	3	135	45	78	34
7–11 m.	215	91	116	39	38	3	44	10	28	9
1–3 yr.	286	176	140	51	53	4	53	14	45	24
4–14 yr.	248	128	89	32	26	0	45	1	27	12
15 yr. or more	70	31	39	6	7	0	14	0	7	5

* Examined. † Positive.

Table 7. *Duration of carrier-state in* 309 Salmonella enteritidis *patients*

Period (days)	Localization of bacteria: Faeces		Urine		Blood	Respiratory tract
	No. of patients	%	No. of patients	%	(no. of patients)	(no. of patients)
–10	112*	37·7	38*	27·5	14*	6*
11–30	110	37·1	43	31·2	9	9
31–60	41	13·9	34	24·6	2	8
61–90	15	5·1	13	9·4	0	8
91–120	8	2·7	3	2·2	0	3
121–150	2	0·7	4	2·9	0	1
151 and more	8	2·7	3	2·2	0	2
Total	296	100·0	138	100·0	25	37
Maximal period (days)	291		236		60	236

* The majority of these patients were examined 1–3 times only.

Since in many cases it was difficult to establish the onset of the infection on the basis of the clinical picture, especially in children whose original disease still persisted at the time of their infection with *S. enteritidis*, it was agreed to consider the day on which the first positive culture from any material was obtained as the probable onset of infection. Table 7 shows data concerning the periods of carrier-state in 309 patients, counting from the probable onset of infection.

As might be expected, patients were positive in the blood for the shortest time,

and only two showed positive blood cultures after the thirtieth day. It is of interest that in four patients positive blood cultures were obtained between 7 and 22 days after the temperature had fallen to normal. In the faeces and urine over 80 % and in the respiratory tract over 60 % were negative after 2 months, but there were a few who continued as carriers for a much longer period. It should be noted that the figures given as maximal periods in Table 7 are not final, as the investigations are not yet complete.

Origin and character of the epidemic

Epidemiological investigations show that, of the total number of salmonella infections diagnosed among patients in the childrens' wards, 12 % may be considered as primary infections, brought from outside ('index cases'), and the remaining 88 % may be considered as hospital infections. The following facts confirm this:

(1) The infections were concentrated in six children's wards, whereas in the remaining thirty-one children's and infectious-diseases wards within the area investigated, although the hospital cases of diarrhoea were examined for salmonellosis, no infections were found.

(2) More than half the cases were admitted to hospital with such diagnoses as pneumonia, influenza, otitis, rheumatic fever, etc., and only 1–3 weeks after admission an unexpectedly high fever or gastro-intestinal disorder occurred. In many of these children the first series of examinations of faeces, urine etc. was negative, and the bacteria were isolated only 1–3 weeks after admission to hospital.

(3) In the area of the epidemic, children with diarrhoea outside hospital were frequently tested bacteriologically for salmonellas; of a total of 151 examined, *S. enteritidis* was found in only three children, of whom two had had previous contact with infected persons.

(4) Of 346 members of the families of the infected children tested 1–3 times, only ten were carriers, and nine of these only after the return of the convalescents to their homes.

(5) In almost all hospital foci, the pattern of infections was intermittent, comprising 3–4 groups of simultaneously occurring infections, with 1-, 2- or 3-week periods of absolute or almost absolute freedom from infections.

It would be difficult to explain all these facts if it is supposed that the infection was endemic in the area examined.

It was possible to exclude the likelihood of the spread of infections by food as prepared in hospital milk kitchens, because (*a*) almost 25 % of those infected had not had their food in such way, (*b*) no carriers were found among the kitchen personnel, despite repeated tests, (*c*) the quality of foods, especially after the introduction of a strict sanitary regime, gave no cause for complaint, (*d*) the central milk kitchen of the Children's Hospital in Lublin supplied three infants' wards with food, yet infections occurred in only one of them.

In the majority of cases it was also possible to exclude direct or indirect contact with infected animals. Towards the end of the epidemic two strains of *S. enteritidis*

were isolated among 160 pigs examined, but these infections are unlikely to have been the cause of this outbreak.

There is thus no doubt that the infections spread in hospital wards by direct contact from man to man. About 60 % of those infected were children up to 1 year of age, and thus without contact with each other, but infection could have been transferred by the hands of the personnel, linen, dishes, teats of nursing-bottles, thermometers and other objects used in nursing. Bacteriological investigations showed that none of these means of transmission can be eliminated: 20–30 % of these objects examined were found to be contaminated with intestinal bacteria (*E. coli*). It should be also emphasized that *S. enteritidis* was twice cultured from the environment—one strain from a swab taken from the metal bed of a sick child and one from a baby's shirt.

The possibility of air-borne infections

The first to draw attention to the spread of salmonellas as an air-borne infection were Varela & Olarte (1942), who isolated salmonellas from the upper respiratory tract. Neter (1950), during a hospital epidemic, isolated *S. oranienburg* from the throat of one child and *S. choleraesuis* from another. In the hospital epidemic described by Datta & Pridie (1960) *S. typhimurium* was found in the nose and throat of five out of fourteen patients tested. Various types of *Salmonella* have been isolated from the air and dust of hospital wards affected by epidemics (Datta & Pridie, 1960; Van Oye, Richard, Moinet & Van Goethem, 1963; Watt *et al.* 1958). The possibility of experimental infections by the respiratory tract in laboratory animals and the development of specific morbid changes have also been demonstrated (Clemmer *et al.* 1960; Darlow, Bale & Carter, 1961; Tully, Gaines & Tigertt, 1963).

In the present investigations, *S. enteritidis* has been found in the upper respiratory tract in thirty-seven cases (Table 3). No less than 39 % of patients examined three or more times were found to be positive (Table 4) and salmonellas were twice recovered from the air. It should be remembered, however, that the isolation of these organisms from the upper respiratory tract is no proof that this was the portal of entry, and our preliminary observations suggest that the localization of these bacteria in the upper respiratory tract, like bacteraemia and bacteriuria, is a secondary phenomenon, a result of their presence in the intestine. The fact that in the great majority of cases observed the bacteria are initially found in the faeces and only later, sometimes after 20–50 days, in the blood, urine, etc., also seems to support the above suggestion.

Despite these reservations it appears fairly probable that some of the cases arose as an air-borne infection. Various epidemiological observations confirm this. A characteristic feature of the observed epidemics was the ease and speed with which the infection spread. The intensity and extent of the epidemic in many children's wards often exceeded the possibilities of diseases transmitted by the faecal–oral route, even if we make allowances for the highly sensitive population in which the infection spread. It was possible to observe sometimes that even a single entry of infection into the wards caused in 1–3 days a wave of a new group of infections,

among children from different rooms who had no contact, directly or indirectly. That some of the observed cases were air-borne is borne out by the fact that routine hygienic measures successfully applied in other foci of alimentary infections, over a long period of time, failed to inhibit the spread of this epidemic. Other authors have also drawn attention to the ineffectiveness of hygienic measures in similar outbreaks (Datta & Pridie, 1960; Neter, 1950; Seeliger & Maya, 1964).

It should be noted that pathological changes, such as inflammation of the throat, lungs, bronchi, etc., were observed in twenty-two throat carriers. In one girl aged 6 months who had not been examined during her illness, salmonellosis was recognized *post mortem* and *S. enteritidis* was isolated from lung tissue taken at autopsy. Other authors (Peluffo, 1964; Saphra & Winter, 1957; Swicowa *et al.* 1962) have also noted symptoms of acute inflammation of the throat in infants with salmonellosis.

To sum up, although the problem of air-borne infection in the spread of salmonellosis needs further investigation, such a method of spread must be considered when methods of control are being planned. Experience gained during the epidemic described confirms the opinion that only such measures as the using of protective masks by the personnel, regular airing of rooms and disinfection of the air (by means of mercury lamps and lactic acid), strict isolation of throat-carriers, etc., together with other hygienic measures, affect the course of a hospital epidemic.

Other epidemiological problems

During the epidemic many cases of infection in newborn infants aged from 2–7 days were observed. When considering the routes of their infection the following possibilities must be taken into consideration: (1) Haematogenous infection from mother-carriers; (2) infection from mother-carriers during or after birth; (3) infection from carriers among the personnel; (4) transmission of bacteria from child to child through hands, napkins or objects used in nursing care; (5) air-borne infection.

In the literature each of these possibilities has its followers and opponents (Abramson, 1947; Neter, 1950; Seeliger & Maya, 1964; Sickenga, 1964; Watt *et al.* 1958). In the epidemic described it was not possible to eliminate any of these modes entirely. Among both mothers and personnel there were several carriers who might be the source of infection for the newborn. On the basis of laboratory investigations and analysis of the entire material it appears, however, that the chief role was played by the transmission of bacteria by the insufficiently disinfected hands of the personnel (38 % of swabs taken from their hands were contaminated with intestinal bacteria), and by air-borne infection.

Transmission of bacteria is in large measure facilitated by the high resistance of *S. enteritidis* in the external environment. Ten positive samples of faeces selected at random were dried and stored at room temperature; from 5, positive cultures were obtained during 3–7 weeks, from 3, during 8–11 weeks, and from 2, up to 13 weeks. These faeces were sampled both in the acute phase of the disease and in the period of convalescence, they came from infants and older children.

Neyman, Stabrowski & Wiza (1954) report an even longer period of the persistence of *S. enteritidis* in dried faeces.

All the investigated strains were highly sensitive to disinfectants (chloramine, sterinol). Inactivation occurred after the action of 0·2 % solutions for 2–5 min.

For obvious reasons the establishment of the incubation period was very difficult. On the basis of various preliminary observations it may be supposed that it is short—several children fell ill 12–24 hr. after exposure to infection. This question, however, requires further investigation.

DISCUSSION

The result of human contact with *S. enteritidis* may be: (1) generalization of bacteria in the organism of the infected person, with accompanying serious clinical syndromes; (2) localization of the bacteria in the intestinal tract with mild enteric disorders; (3) inapparent cases limited to the intestinal phase of the infection; (4) no infection. As has been mentioned, observations made during these investigations suggested that the place of primary localization of *S. enteritidis* in the human organism is probably the intestines, and thence, for various reasons, in some cases the bacteria penetrate to other systems. At present it is difficult to define precisely all the factors which determine the overcoming of the protective barriers of the alimentary tract and the development of one or another form of the disease. In the literature there are data on the effect of such factors as the virulence of particular strains, the natural resistance of the host, the dose of transmitted bacteria, acquired immunity, age and debilitating factors (McCullough & Eisele, 1951, Sickenga, 1964).

Our investigations show that the younger the children, the more frequent were positive findings outside the alimentary tract, and the more severe was the course of the disease. Whereas in children aged up to 1 year salmonellas were detected parenterally in almost 85 % of those examined, in children aged 4–14 years and in adults they were found in the upper respiratory tract in only one patient and were not found in the blood in any. From thirty-nine samples of urine taken from adults salmonellas were found in only six. The effect of debilitating factors was also very clear. Almost 80 % of the infected children were between 10 % and 30 % underweight, 8 % were premature infants and 86 % had intercurrent disease. These last had mostly been treated, before their salmonella infection, with large doses of various antibiotics, which doubtless affected the biocoenosis of the intestines. The period of bacterial excretion was much longer in infants than in older children: a large percentage of infants were positive in faeces, urine or the upper respiratory tract for 5–8 months, but the older children usually became negative in 5–15 days. These data agree with those of Crainz *et al.* (1960), Mihalyfi *et al.* (1961), McCullough & Eisele (1951), Neter (1950), Stahn (1963) and Szanton (1957).

From the epidemiological viewpoint it is clear that the generalized forms, in which the organisms are found in the throat or the urinary tract, are the most dangerous. Since these forms are met mainly in infants, who are in addition most susceptible to infection, this type of salmonellosis is primarily an infection of

infants in hospital, while older persons may be only chance, temporary and sometimes 'blind' links in the epidemic chain. It is characteristic that, of 159 members of the families of forty-nine children discharged from hospital as persistent carriers, only three became infected, one adult and two children aged 4 and 7. It is worth noting here that in the great majority of these families the hygienic conditions were unsatisfactory, and contacts had not been previously vaccinated against typhoid and paratyphoid fevers, which has been shown by Levine, Enright & Ching (1962) to confer some heterologous immunity against salmonellas of groups B and D.

The great difficulties encountered in the control of this outbreak seem to have been due to a combination of many factors, the most important of which were the high susceptibility of children to infection, the tendency to generalization of the infection, the persistent excretion, sometimes for many months, in faeces, urine and upper respiratory tract, the resistance of the organism to drying and to antibiotics and the short incubation period. All these factors contributed to an outbreak in which there were many seriously ill patients, with a high mortality rate amongst the new-born and the infants.

SUMMARY

During 9 months the author observed 309 cases of infection with *Salmonella enteritidis*, of which 88 % were hospital infections in six childrens' wards of three different hospitals. The foci were connected epidemiologically. In 97·7 % of those examined, the bacteria were detected in faeces, in 68 % in urine and in 27·5 % in the upper respiratory tract. Bacteraemia was found in 17·2 %. The bacteria were also detected in two air-samples and in two swabs from linen and furniture. All isolated strains were highly resistant to antibiotics and to drying, but sensitive to the action of chloramine. In infected patients with parenteral localization of bacteria, the agglutination test with specific gm antigen was positive in 80 % of cases. Many patients were persistent carriers for 5–10 months. In adults a short faecal carrier state was usually observed. The epidemic spread by contact, though in some cases it was probably air-borne. Such epidemics are very difficult to bring to an end.

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The development of skin resistance and hypersensitivity following inactivated vaccinia virus vaccines in rabbits

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INTRODUCTION

Skin resistance or skin immunity to vaccinia virus is a well recognized phenomenon, although its mechanism is not understood. It is a matter of common observation that it can be more difficult to produce a vaccination 'take' in previously vaccinated than in non-vaccinated individuals. Though development of skin immunity after infection with live vaccinia virus is well established, there is considerable difference of opinion concerning the development of skin immunity as a result of immunization with inactivated vaccinia virus.

According to some investigators (Andrewes, Elford & Niven, 1948; Amies, 1961; RamanaRao, 1962) inactivated vaccinia virus vaccines do not produce an increase in skin immunity. There are others who consider that inactivated vaccinia virus can induce skin immunity but that there is no apparent relationship between this and the level of circulating antibody, (Kaplan, 1960; Beunders, Driessen & van den Hoek, 1960). Thirdly, it is believed that inactivated vaccinia virus can produce both circulating antibody and skin immunity (Parker & Rivers, 1936; Collier, McClean & Vallet, 1955). In order to investigate the development of skin resistance following immunization with inactivated vaccinia virus, and the relationship of this skin resistance to circulating antibody, it is necessary in the first place to define skin immunity, and on this basis to use an appropriate method for measuring it. It seems likely that different concepts of the nature of skin immunity and therefore the utilization of different methods for measuring it have contributed to the confused picture outlined above. Skin immunity can be defined as the resistance of the skin to the initiation of a focus of multiplication by a given inoculum of virus. Thus for each experimental animal one can determine, by a virus titration in the skin, the minimal inoculum of virus which will initiate a focus of multiplication. With this quantitative method difficulties of interpretation arise in deciding whether a skin reaction to virus represents a focus of multiplication or whether it represents a hypersensitivity reaction to the inoculated virus without multiplication necessarily having taken place. This difficulty can be overcome by using a control series of inactivated virus inocula in each animal, the quantity of virus in each inactivated dilution being identical with that in the corresponding live dilution. A larger reaction of longer duration at the site of challenge with live virus would indicate that multiplication had taken place. This method was used in the present study and is described in detail under Methods.

An alternative qualitative method for measuring skin immunity has been

frequently used which involves challenging the skin with a single dose of high-titre virus and recording the results as a primary, accelerated, or immune response, or by some similar notation. By this method conversion from primary type reaction to an accelerated reaction may be interpreted as an index of skin immunity. Such an interpretation could possibly imply that delayed-type hypersensitivity is an important factor in skin immunity. This would be based on the possibility that a primary reaction and an accelerated reaction are similar foci of viral multiplication, the accelerated reaction being accelerated because the subject possessed residual delayed hypersensitivity. This raises another question, namely, is there any relationship between the degree of skin resistance and the degree of delayed-type hypersensitivity? The present investigation was undertaken in an attempt to clarify these problems. Two questions were asked:

(i) Is there any increase in skin immunity following immunization with inactivated vaccinia virus?

(ii) If skin immunity does develop, is this related to either the level of circulating antibody or the degree of delayed hypersensitivity?

METHODS

Skin tests were performed in the rabbits which had been used for the study of antibody responses to inactivated vaccinia virus (McNeill, 1965). Methods for preparation of the vaccines, titration of infectivity, titration of virus neutralizing antibody, and immunization of rabbits were as previously described. Skin testing was performed in each rabbit 2 weeks after a second dose of inactivated vaccine. Sera were collected before inoculation of skin-testing virus and titrated for neutralizing antibody.

Skin testing

Rabbits were tested by intradermal inoculation of dilutions of active and inactivated virus in the shaved skin of their backs. Each inoculum was 0·1 ml. Preliminary experiments were performed in order to determine the end-point range with serial tenfold dilutions of live virus in normal and previously vaccinated rabbits. From these experiments it was concluded that a suitable range of titres for test virus was 10^4—10 plaque forming units (pfu) per inoculum.

Preparation of skin-testing virus

Vaccinia virus grown on Hep2 cells was partially purified by one cycle of differential centrifugation. Virus was diluted to 10^5 pfu/ml. and divided into two equal parts, one of which was inactivated by heat at 60° C. for 1 hr. Two tenfold, followed by two fourfold dilutions were made of both the active and inactivated suspensions. The fourfold dilutions were made in order to give a more precise end-point in the skin titrations. Dilutions of active virus were dispensed in 1 ml. amounts and stored at − 70° C., and dilutions of inactive virus were dispensed in 20 ml. amounts and stored at 4° C. Each rabbit was inoculated with each dilution of active and inactive virus, active virus being inoculated on the right side of the back, and inactive virus on the left side. The inactive virus not only served as a control for

the hypersensitivity reaction to the quantity of virus inoculated, but it also allowed an estimation of the degree of hypersensitivity by determining the highest dilution of inactivated virus which gave rise to a reaction. The diameter of induration following each inoculation was recorded daily for 5 days. A reaction to active virus which persisted at least 24 hr. longer than the reaction to the identical quantity of inactive virus, and which gave an indurated lesion greater by 2–3 mm. than the lesion from the inactive virus, was interpreted as evidence that some viral multiplication had taken place.

RESULTS

Table 1 gives the complete results for a normal rabbit and an immunized rabbit to illustrate the interpretation of the skin tests and the measurement of skin resistance and hypersensitivity.

Table 1. *The diameter of induration in mm. following intradermal inoculation of dilutions of active* (A_1–A_5) *and inactive* (I_1–I_5) *virus in a normal rabbit and in a rabbit previously immunized with inactivated vaccine*

Normal rabbit

Titre...	10^4 pfu/dose		10^3 pfu/dose		10^2 pfu/dose		25 pfu/dose		6 pfu/dose	
Day	A_1	I_1	A_2	I_2	A_3	I_3	A_4	I_4	A_5	I_5
1	7	—	4	—	—	—	—	—	—	—
2	9	—	7	—	5	—	3	—	2	—
3	12	—	9	—	8	—	5	—	3	—
4	15	—	11	—	10	—	7	—	4	—
5	9	—	6	—	7	—	7	—	5	—

Immunized rabbit

Titre...	10^4 pfu/dose		10^3 pfu/dose		10^2 pfu/dose		25 pfu/dose		6 pfu/dose	
Day	A_1	I_1	A_2	I_2	A_3	I_3	A_4	I_4	A_5	I_5
1	8	4	3	4	2	2	—	—	—	—
2	11	2	5	2	4	—	—	—	—	—
3	15	—	7	—	2	—	—	—	—	—
4	15	—	7	—	2	—	—	—	—	—
5	10	—	6	—	2	—	—	—	—	—

It can be seen that the normal rabbit reacted to every dilution of active virus, but not to any of the dilutions of inactive virus. It is therefore recorded as having a skin resistance of 0, and a degree of hypersensitivity of 0. Six normal rabbits were tested, and all reacted to the highest dilution of live test virus. The immunized rabbit, on the other hand, showed both skin resistance and hypersensitivity. The A_3 lesion in this rabbit was the lowest dilution to give a reaction, and this was clearly greater than the I_3 lesion. Taking the lowest dose (A_5) as unity, according to the previously stated dilutions this represents 16 times the amount of virus required to produce a lesion in normal rabbits. The rabbit was therefore recorded

as having a skin resistance of 16. To record the degree of hypersensitivity, a reaction to the most concentrated suspension only (I_1) was recorded as 1 and to the other dilutions as 10, 100, 400 and 1600 respectively. This rabbit, which reacted to I_3, was recorded as having a degree of hypersensitivity of 100. These figures are obviously only relative, but nevertheless serve a useful purpose in that they provide at least a semi-quantitative estimation of two factors which are difficult to measure accurately.

Using this method the degree of skin resistance was determined for ninety-seven rabbits which had received two doses of various inactivated vaccinia virus vaccines. These experiments showed quite clearly that skin resistance to vaccinia virus can be increased by the administration of inactivated vaccine, although in many animals this increase was minimal, requiring only a fourfold increase in the amount of challenge virus to overcome it. The number of different vaccines used, and the small number of rabbits which received each vaccine, makes a calculation of the percentage of rabbits which developed skin immunity quite meaningless. However, as it has been shown that the most important factor in the immunogenicity of inactivated vaccinia virus in terms of development of neutralizing antibody was the dose of antigen in the vaccine (McNeill, 1965) the question arises as to whether development of skin resistance is also related to the dose of antigen. Table 2 shows the relevant data from thirty-five rabbits. No distinction is made in this table between degrees of skin resistance.

Table 2. *Relationship between virus concentration in vaccine (pfu/0·5 ml. before inactivation) and development of increased skin resistance*

Virus content of vaccine (pfu/0·5 ml.)	Incidence of skin resistance
$2{\cdot}8 \times 10^8$	12/12
9×10^7	3/3
7×10^7	2/4
3×10^7	1/4
$1{\cdot}8 \times 10^7$	1/4
6×10^6	0/4
4×10^6	1/4

It appears therefore that both skin resistance and the type of antibody response are dependent upon the dose of antigen used to immunize.

Is the degree of skin resistance related to the level of circulating antibody? Figure 1 shows the relationship in the form of a scatter diagram drawn from data obtained by tests on ninety-seven rabbits. This shows a definite although broad relationship between these factors. Figures 2 and 3 show that there is no relationship between the degree of hypersensitivity and either the degree of skin resistance or the level of circulating antibody.

Unusual skin reactions

An unexpected feature of the skin testing was that some rabbits developed very severe skin reactions at the sites of inoculation of live virus. These severe reactions were characterized by the development within 36–48 hr. after inoculation of a

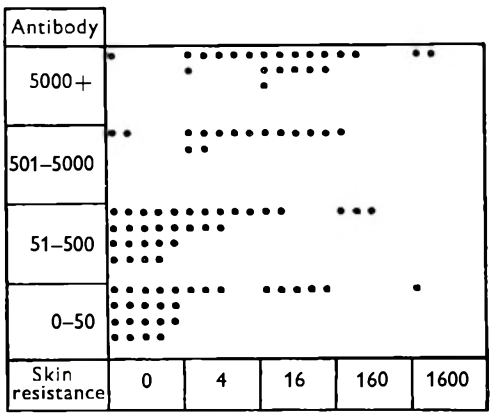


Fig. 1. A scatter diagram to show the relationship between virus neutralizing antibody and skin resistance to vaccinia virus following immunization with inactivated vaccinia virus vaccines.

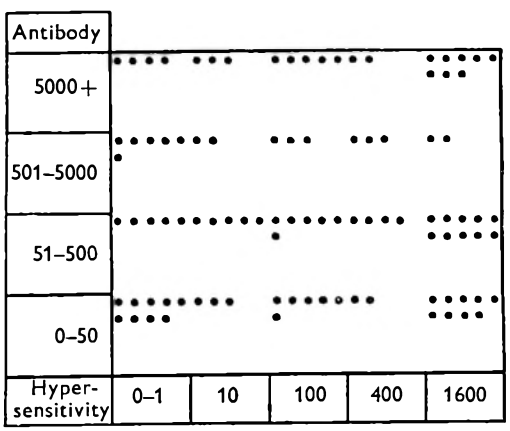


Fig. 2. A scatter diagram to show the relationship between virus neutralizing antibody and delayed hypersensitivity to vaccinia virus following immunization with inactivated vaccinia virus vaccines.

circumscribed indurated lesion with a surrounding area of intense erythema. By the third or fourth day the indurated area had become haemorrhagic and necrotic. Plate 1*a* shows one of these reactions on the sixth day, and Plate 1*b* a normal reaction to vaccinia virus at this stage for comparison. These severe reactions were not seen at any of the sites of challenge with inactivated virus. Histologically the predominant feature was an intense cellular infiltrate of the skin and subcutaneous tissue. This infiltrate was mainly composed of monocytes with a few eosinophils and plasma cells. Islands of perivascular lymphocytic infiltration were seen in some sections.

The most severe reactions occurred in the series of experiments with vaccines prepared by using different inactivating agents and different degrees of inactivation (McNeill, 1965). There was an obvious difference in the severity of the reaction in that rabbits immunized with hydroxylamine-inactivated vaccines showed much more severe reactions than those immunized with either formalin or heat-inactivated vaccines. This is shown in Table 3.

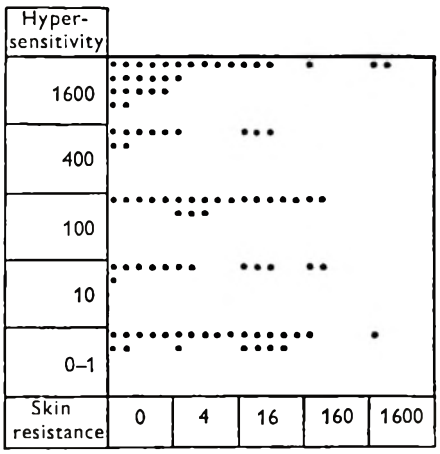


Fig. 3. A scatter diagram to show the relationship between skin resistance and delayed hypersensitivity to vaccinia virus following immunization with inactivated vaccinia virus vaccines.

Table 3. *Average diameter in mm. of the indurated lesion following I.D. inoculation of* 10^4 *pfu virus in rabbits showing the severe reaction*

(Measurements 5 days after inoculation.)

Formalin (nine severe reactors)	Hydroxylamine (seven severe reactors)	Heat (two severe reactors)
13	25	15

Table 4. *Incidence of severe reactions following skin challenge in rabbits immunized with inactivated vaccinia virus vaccine suspended in either buffer or polyvinylpyrrolidone*

Vaccine base	Severe reactions	Percentage
PVP	21/72	30
Buffer	2/25	8

The presence of polyvinylpyrrolidone (PVP) in the immunizing vaccine appeared to be a contributory factor. Table 4 shows the incidence of severe reactions in rabbits receiving PVP vaccines compared with those receiving vaccines suspended in buffer alone.

There was a tendency for rabbits with lower levels of circulating antibody to show this type of reaction, as shown in Table 5.

Table 5. *Incidence of severe reactions in relation to the level of circulating antibody*

Neutralizing antibody titre	Severe reactions	Percentage
0–20	8/19	43
21–200	8/29	28
201–2000	6/24	25
2000+	1/25	4

DISCUSSION

The results of these experiments indicate that inactivated vaccinia virus vaccines can produce an increase in skin resistance to live virus in rabbits, and that the development of this skin resistance is dependent upon the quantity of antigen in the vaccine. Furthermore, the degree of skin resistance is related to the level of circulating neutralizing antibody and not to the degree of delayed hypersensitivity. These results are in apparent conflict with results reported by several other workers which were referred to in the introduction to this paper.

In considering these discrepancies there are three factors which should be considered. First, it has been shown in Table 2 that development of skin resistance is dependent upon the dose of antigen in the vaccine, and that regular production of skin resistance was produced only by vaccines containing approximately 10^8 pfu/dose infectivity before inactivation. Such concentrations of virus are greater than were used by many other workers. Secondly, it can be seen from Fig. 1 that the relationship between skin resistance and antibody titre is not a precise one, and is therefore likely to be apparent only when relatively large numbers of animals are studied. Experiments with sub-optimal doses of antigen in small numbers of animals could easily result in only a few animals developing skin resistance, and no apparent relationship between this and the titre of antibody. Thirdly, as previously explained, the method of assessing skin resistance could be important. It is interesting to note that several authors who found no relationship between skin resistance and titre of antibody had measured skin resistance by a qualitative method. The observations reported here show that there is no relationship between the degree of hypersensitivity and either the level of circulating antibody or the degree of skin resistance, and offer a possible explanation of why qualitative estimation of skin immunity in which hypersensitivity reactions are intimately involved may not show any relationship with levels of circulating antibody.

The severe florid reactions which were shown by some animals to challenge virus were quite unexpected. The rabbits which showed this type of reaction to live virus did not show any abnormality in their response to inactivated skin-testing virus. This may have been due to there being an insufficient quantity of antigen, since viral multiplication was necessary to provide an adequate stimulus for the development of this reaction. Of the rabbits showing severe reactions to live virus, 50 % reacted to all dilutions of inactive virus, compared with 30 % of normally reacting

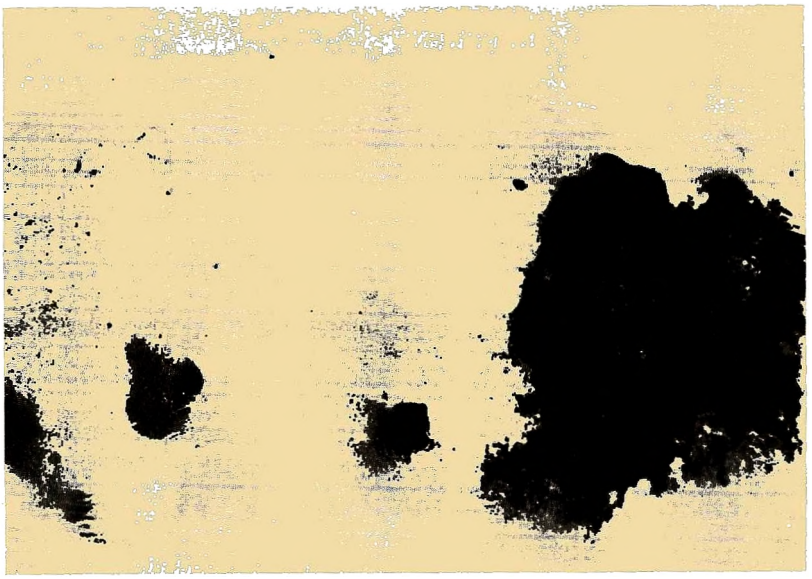
rabbits. The remaining 50% of severe reactors showed varying degrees of hypersensitivity to the inactivated test virus. This reaction was therefore more than a quantitative exaggeration of the normal reaction, it was also qualitatively different in that a pronounced 'vasculo-necrotic' element was present. The reactions were most severe in rabbits previously immunized with hydroxylamine-inactivated vaccines; the method of inactivation which theoretically caused least damage to the immunizing viral antigens. They were associated with the presence of PVP in the vaccines, this being the only manifestation of any adjuvant activity by this substance which was found (McNeill, 1965). The rabbits which developed the reaction tended to have lower levels of circulating antibody. Whatever the precise classification or aetiology of this severe reaction may be, it could be of practical importance particularly from the point of view of using inactivated vaccines before live vaccine. It is clearly possible that under certain circumstances, reactions to live vaccine after immunization with inactivated vaccine could be worse than primary vaccination with live vaccine alone.

Several reports of unusual skin reactions to vaccinia virus after immunization with inactive vaccine have appeared in the literature, e.g. Bussel & Mayzner (1930) reported two children who developed markedly erythematous and indurated lesions after Jennerian vaccination following three inoculations of formalin-inactivated virus. The lesions were much more severe than those resulting from the use of the same vaccine in previously unimmunized children. Weil & Gall (1940) noted that two rabbits showed a much more necrotic reaction to challenge virus after immunization with inactivated vaccine than was usual. It is interesting to note that in contrast to the other rabbits in the experiment one of these severe reactors had no detectable circulating antibody, and the other had a very low titre. Ehrengut (1959) described a peculiarity of the vaccination reaction in 4% of children who had previously been immunized with inactivated vaccine. This 'Hügelreaktion' was interpreted as an abnormal hypersensitivity reaction to the virus. RamanaRao (1962) was unable to demonstrate any quantitative increase in skin resistance following either live or inactivated vaccines, but intradermal challenge with live virus differentiated rabbits which had been immunized with inactivated vaccine from those immunized with living virus. The former showed lesions with necrosis, whereas the latter did not.

SUMMARY

A study was made of the development of skin resistance and delayed hypersensitivity to vaccinia virus after immunization with inactivated vaccinia virus in rabbits. These vaccines are shown to increase skin resistance, and it is also shown that the development of this resistance is dependent upon the virus content of the vaccine. Skin resistance is shown to be related to the titre of circulating antibody rather than to the degree of delayed hypersensitivity. Possible reasons for conflicting reports in the literature on this subject are discussed.

An unusual skin reaction was seen in some animals when challenged with live virus after immunization with inactivated vaccine. The nature of these reactions is discussed.



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I wish to thank the National Fund for Research into Poliomyelitis and Other Crippling Diseases for their support in the form of a Research Fellowship during the tenure of which this investigation was made.

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EXPLANATION OF PLATE

(*a*) The back of a rabbit, showing a severe type of reaction following intradermal challenge with live vaccinia virus. Photograph taken 6 days after challenge.

(*b*) The back of a rabbit, showing a normal reaction to vaccinia virus. Photograph taken 6 days after challenge.

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The influence of unheated guinea-pig serum on the neutralization of Semliki Forest virus by various antisera

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INTRODUCTION

The presence of fresh unheated animal serum in the neutralization mixture has been reported to reduce the persistent fraction of western equine encephalitis (WEE) virus (Dulbecco, Vogt & Stickland, 1956). Similarly, Sabin (1950) reported that unheated normal serum potentiates the neutralization of dengue virus. Hashimoto & Price (1963) demonstrated that fresh unheated guinea-pig serum did not modify the kinetics of the neutralization of Japanese B encephalitis virus.

In the course of studies of the modifications of the antibody binding capacity of normal and chemically modified Semliki Forest virus, the optimal experimental conditions of Semliki Forest virus neutralization had to be searched for.

The different neutralization curves obtained with different antisera, and the modifications of these curves resulting from addition of unheated guinea-pig serum or of EDTA to the neutralization mixture, are reported here.

MATERIALS AND METHODS

Virus

Purified and crude suspensions of Semliki Forest virus were used. The preparation of purified virus will be described elsewhere (Osterrieth & Calberg-Bacq (to be published). The crude virus suspension was a 10 % (w/v) suspension of infected unweaned mouse brains in saline. After homogenization, the suspension was clarified by centrifugation at 3000*g* for 30 min. The infectious titre of the suspension was 10^{10} plaque-forming units (pfu)/ml. The suspension was stored at $-70°$ C.

Solutions

PBS: Phosphate buffered saline (0·15 M-NaCl, 0·01 M phosphate buffer pH 7·2).

GBSS: Tris Gey's solution of Porterfield (1960) pH 7·6 to which was added 0·1 % (w/v) bovine plasma albumin (Armour fraction V).

EDTA stock solution: 0·1 M ethylene diamine tetraacetic acid in distilled water adjusted to pH 7·0 with M-NaOH.

* Chercheur qualifié du F.N.R.S.

Infectivity assays

Chick embryo-cell monolayers were used. The plaque technique that was used will be described elsewhere (Osterrieth & Calberg-Bacq, to be published).

Antisera

Hyperimmune rabbit serum

A rabbit was injected with 5 ml. of the crude suspension of Semliki Forest virus on days 1, 4, 12, 16, 20 and 24. After the second injection, the rabbit fell ill and developed paralysis of the hind legs. The animal was bled on the 45th day. The serum was centrifuged at 105,000*g* for 30 min. and the supernatant was stored at −20° C. Before use, the serum was diluted 1/10 in PBS and heated at 56° C. for 30 min.

Absorbed hyperimmune rabbit serum

Ten ml. of rabbit antiserum was mixed with the pellet obtained by low-speed centrifugation of 10 ml. of 20 % w/v suspension of normal unweaned mouse brain in saline. The mixture was incubated at 37° C. for 1 hr. and then overnight at 4° C. The next day, the mixture was clarified by a 35 min. centrifugation at 1500*g* and then centrifuged at 105,000*g* for 30 min. The supernatant, called absorbed serum, was stored at −20° C. Before use, it was diluted and inactivated as the rabbit antiserum.

Rabbit anti-mouse brain serum

A 10 % (w/v) normal unweaned mouse brain suspension in saline was clarified by centrifugation at 1500*g* for 30 min. The supernatant was used for immunization and 5 ml. were injected intramuscularly, on day 1, 5, 9, 13, 17 and 20 to two rabbits. The animals were bled on the 42nd day. The serum was stored at −20°C. Before use, the serum was diluted and heated as previously described.

Ascitic fluid from hyperimmunized mice

Crude suspension of Semliki Forest virus was mixed with an equal volume of Freund complete adjuvent (Difco), and 0·2 ml. of this mixture was injected into the peritoneal cavity of mice, at 7-day intervals. At the time of the first injection the mice were 4 weeks old. The virus suspension used for the first two injections had been inactivated by a 7-day storage at 4° C. after addition of formalin (0·5 %, w/v). One week after the sixth injection the ascitic fluid was harvested. The average yield per mouse was 7·5 ml. After a few hours at room temperature a slight coagulum appeared, which was removed by low-speed centrifugation. The supernatant was opalescent and was centrifuged at 105,000*g* for 1 hr. The resulting supernatant displayed a fatty top layer that was discarded, and a clear lower layer that was stored at −20° C. On thawing, a slight coagulum appeared once more, which was removed. Before use, the fluid was heated at 56° C. for 30 min.

Immune guinea-pig serum

A guinea-pig was injected intracerebrally with 0·5 ml. of Semliki Forest virus crude suspension. Two weeks later, 1 ml. of the same suspension was injected intramuscularly. The animal was bled at the end of the third week. The serum was stored at $-20°$ C. Before use, it was heated at 56° C. for 30 min.

Normal guinea-pig serum

Pools were made with the sera of twenty normal male guinea-pigs. These pools were stored in 2 ml. volumes at $-20°$ C.

Neutralization tests

Two or fourfold serial dilutions of serum (or ascitic fluid) were made in PBS, in PBS containing 1% (v/v) normal unheated guinea-pig serum, in 0·002 M EDTA in PBS, in 0·01 M EDTA in PBS, in 0·002 M (or 0·01 M) EDTA in PBS containing 1% (v/v) unheated normal guinea-pig serum, and in PBS containing 1% (v/v) heated normal guinea-pig serum. To 0·5 ml. of these serum dilutions were added equal volumes of Semliki Forest virus suspension. The virus suspensions, either crude or purified, were always diluted in GBSS so as to give, in the neutralization mixture, a virus concentration of about 10^5 pfu/ml. The virus titre was determined from virus–PBS mixtures. Serum–virus mixtures and virus controls were kept at 0° C. for 3 hr. The mixtures were then diluted 1/10 in GBSS and maintained at 0° C., awaiting final dilution and plating. The final dilutions (3·16-fold steps in GBSS) were immediately inoculated to monolayers of chick embryo cells (1 ml. inoculum per Petri dish 8 cm. diameter) previously washed once with 4·5 ml. of GBSS. Adsorption was carried out at room temperature for 90 min. At the end of the adsorption period the excess of fluid was sucked out and the overlay poured.

RESULTS

Kinetics of the neutralization

These experiments were carried out with rabbit absorbed antiserum and purified virus. Final concentrations of serum in the neutralization mixtures were $10^{-3·3}$, $10^{-3·8}$, and $10^{-4·3}$. Incubation times at 0° C. were 15, 30, 60, 90 and 120 min. The results are given in Fig. 1. The curve C showed a descending part and a relatively horizontal part. The descending part is nearly a straight line, compatible with first-order reaction. The horizontal part tends to become parallel to the abscissa, showing that the surviving fraction tends to a minimal value characteristic for each serum concentration, at constant virus concentration. Neutralization appeared almost complete after 90 min. incubation, and 180 min. was chosen as standard incubation time for the other experiments.

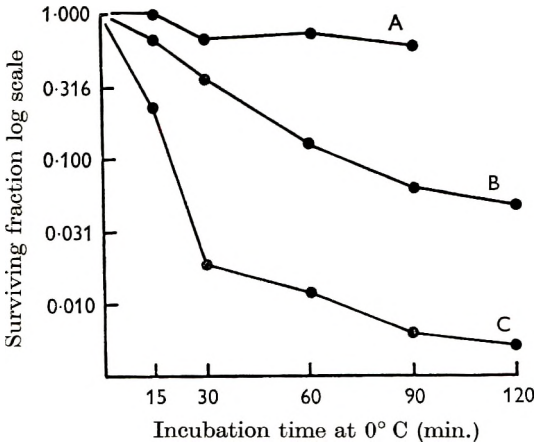

Fig. 1. Kinetics of the neutralization of purified Semliki Forest virus by absorbed hyperimmune rabbit serum. Final concentration of the serum in the neutralizing mixture: curve A $10^{-4\cdot3}$, curve B $10^{-3\cdot8}$ and curve C $10^{-3\cdot3}$.

Neutralization of purified Semliki Forest virus by rabbit absorbed antiserum

Figure 2 shows the neutralization curve obtained when a constant dose of Semliki Forest virus was incubated for a constant length of time with decreasing doses of rabbit absorbed antiserum, and the modifications which were obtained when 0·5% (v/v) heat-inactivated normal guinea-pig serum, 0·5% (v/v) unheated guinea-pig serum, EDTA (0·001 M and 0·005 M), or EDTA (0·001 M or 0·005 M) together with 0·5% (v/v) unheated normal guinea-pig serum were present in the neutralization mixture. The log of the surviving fraction of the virus was taken as ordinate, the dilution of the antiserum in the neutralization mixture as abscissa. The dose of antiserum that neutralizes 90% of the plaque forming units (pfu), was called neutralizing dose (Fazekas de St Groth, 1961).

When the antiserum concentration rises, the surviving fraction decreases steeply at first, very slowly afterwards. The value of the surviving fraction corresponding to the $10^{-2\cdot3}$ dilution of the antiserum is 0·0032. The neutralizing dose corresponds approximately to the $10^{-4\cdot1}$ dilution of the antiserum.

The presence of 0·001 M and 0·005 M EDTA in the antiserum-virus mixture does not modify the neutralization curve, nor does the presence of 0·5% (v/v) heated normal guinea-pig serum.

When 0·5% (v/v) unheated normal guinea-pig serum is added to the virus–serum mixtures, the neutralization curve is completely transformed. The surviving fraction still decreases at first when the antiserum concentration is raised, yet it quickly reaches a minimum value and finally increases although the antiserum concentration is still increased. The minimum value of the virus survival corresponds to a $10^{-4\cdot1}$ dilution of the antiserum. The surviving fraction obtained with the $10^{-2\cdot3}$ dilution of the antiserum is now 30 times greater than it was before addition of unheated normal guinea-pig serum. Moreover, the neutralizing dose of the antiserum that was $10^{-4\cdot1}$ becomes, in presence of normal unheated guinea-pig

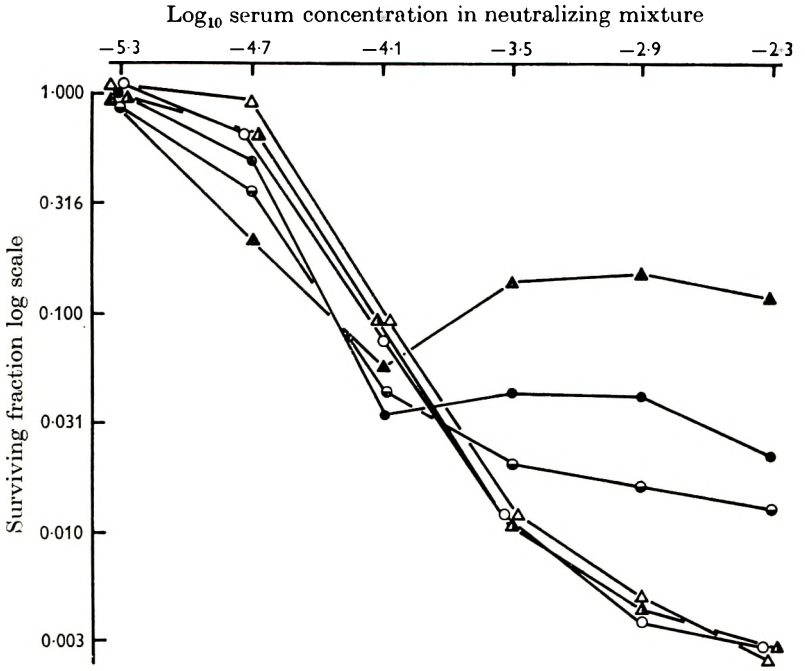

Fig. 2. Neutralization of purified Semliki Forest virus by absorbed hyperimmune rabbit serum. Serum diluent: △, PBS; ◭, PBS containing 1 % (v/v) heated normal guinea-pig serum; ▲, PBS containing 1 % (v/v) unheated normal guinea-pig serum; ○, 0·002 M EDTA in PBS, ◒, 0·01 M EDTA in PBS containing 1 % (v/v) unheated normal guinea-pig serum; ●, 0·002 M EDTA in PBS containing 1 % (v/v) unheated normal guinea-pig serum.

Table 1. *Effect on the virus titre of the presence in the virus diluent of EDTA, complement, pre-immunization rabbit serum and anti-mouse brain rabbit serum*

	Log_{10} of the virus titres expressed in pfu/ml.		
Diluent	No serum	Normal rabbit serum 10^{-2}	Anti-mouse brain rabbit serum 10^{-2}
PBS	4·90	5·66	5·96
PBS + EDTA (10^{-3} M)	4·83	5·70	5·93
PBS + C′ (1/200)	4·92	5·73	5·90

Each column gives the mean results of two experiments.

serum, $10^{-4\cdot4}$, one half of its previous value. When the antiserum concentration is low, unheated normal guinea-pig serum decreases the surviving fraction and potentiates the neutralization slightly. Conversely, when the antiserum concentration is high, unheated normal guinea-pig serum increases the surviving fraction and inhibits neutralization.

When EDTA is added together with unheated normal guinea-pig serum the neutralization curve tends to return to normality although it still shows some

distortion. The neutralization mixture was 0·0014 M in Ca^{2+} and 0·001 M in Mg^{2+}. The 0·001 M concentration of EDTA was thus a little low, but the 0·005 M concentration was sufficient. Addition of EDTA did not completely suppress the action of unheated normal guinea-pig serum as did heating at 56° C. for 30 min.

The serum obtained from our rabbit before immunization had no effect on the virus titre either in the presence of unheated normal guinea-pig serum or in the presence of EDTA (Table 1). There were no pre-immunization non-specific neutralizing substances.

Unheated normal guinea-pig serum alone and EDTA alone had no effect on the virus titre (Table 1).

A second pool of normal guinea-pig serum was used. The same effect was observed.

Neutralization of crude and purified virus by absorbed and non-absorbed rabbit antiserum with and without unheated normal guinea-pig serum

Figure 3 shows the curves obtained: (A) with absorbed antiserum and purified virus, (B) with non-absorbed antiserum and purified virus and (C) with non-absorbed antiserum and crude virus suspension.

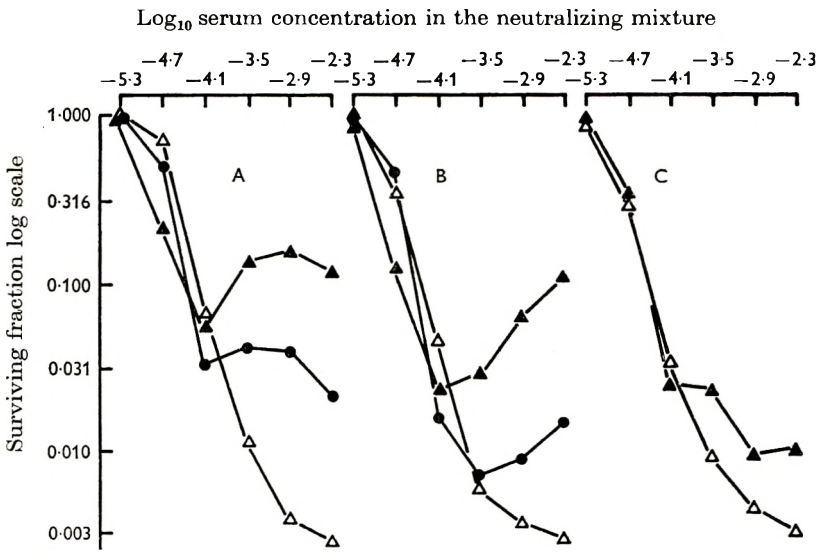

Fig. 3. Neutralization of crude and purified Semliki Forest virus by hyperimmune rabbit serum. Curve A, purified virus, absorbed serum; curve B, purified virus non-absorbed serum; curve C, crude virus, non-absorbed serum. Serum diluent: △, PBS; ▲, PBS containing 1 % (v/v) unheated normal guinea-pig serum; ●, 0·002 M EDTA in PBS containing 1 % (v/v) unheated normal guinea-pig serum.

Neither the use of absorbed serum nor the use of purified virus modified the relation between the surviving fraction and the serum concentration. The effect of unheated normal guinea-pig serum was similar in all three systems although it was increased by the use of purified virus and still further increased when absorbed

antiserum was used together with purified virus. EDTA depressed the action of unheated normal guinea-pig serum on the neutralization by rabbit absorbed antiserum as well as by rabbit non-absorbed antiserum.

No neutralization was obtained with the serum of rabbits which were immunized with a suspension of brains of non-infected unweaned mice, either in presence of EDTA or in presence of unheated normal guinea-pig serum (Table 1). This shows that the immunological modifications induced in the rabbit by repeated injection of normal mouse brain antigens are not, alone, capable of virus neutralization.

Effect of the concentration of unheated normal guinea-pig serum on the neutralization of a constant dose of virus by a constant dose of serum

As only the inhibition of neutralization was measurable with a reasonable accuracy, when rabbit antiserum was used, the influence of the dose of complement was investigated in this zone. In these experiments we used absorbed rabbit antiserum at the final concentration, $10^{-2.9}$. This concentration corresponds to the

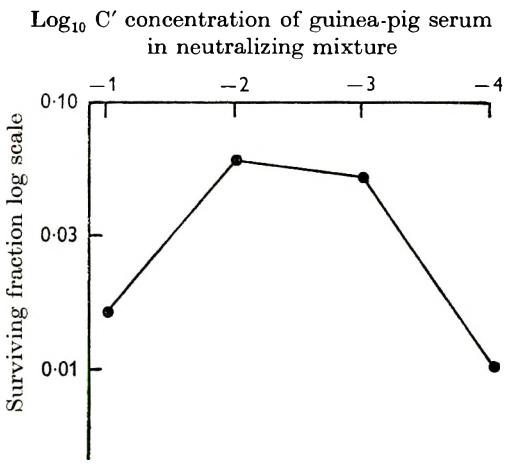

Fig. 4. Effect of the concentration of unheated normal guinea-pig serum on the neutralization of Semliki Forest virus by absorbed hyperimmune rabbit serum. Serum concentration: $10^{-2.9}$.

maximum inhibition of neutralization in presence of 0·5 % (v/v) of unheated normal guinea-pig serum. Figure 4 gives the mean results of three experiments. When the final concentration of unheated normal guinea-pig serum in the neutralizing mixture is raised from 0·01 % to 10 % (v/v) the surviving fraction increases, reaches a maximum value and then decreases. There is an optimal concentration of complement that corresponds to a maximum in the inhibition of neutralization. Above this optimal concentration of complement, the inhibition of the neutralization is less pronounced.

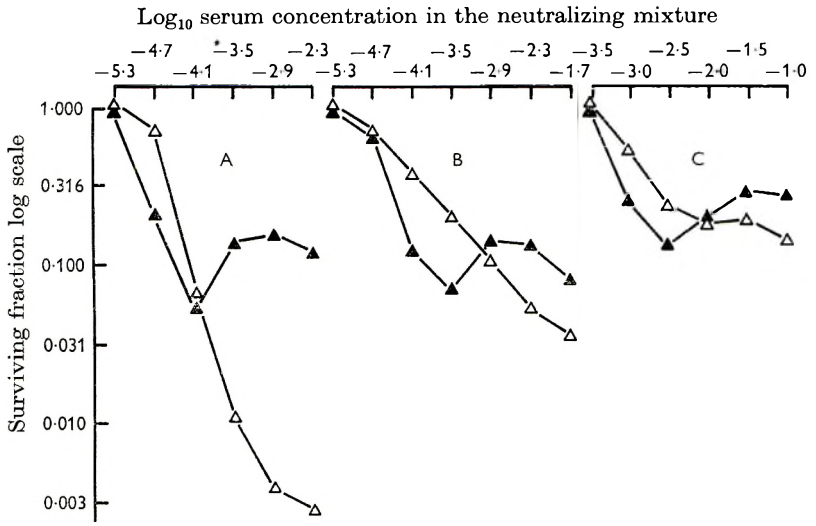

Fig. 5. Neutralization of purified Semliki Forest virus by various antisera. A, Absorbed hyperimmune rabbit serum; B, ascitic fluid from hyperimmunized mice; C, immune guinea-pig serum. Serum diluent: △, PBS; ▲ PBS containing 1 % (v/v) unheated normal guinea-pig serum.

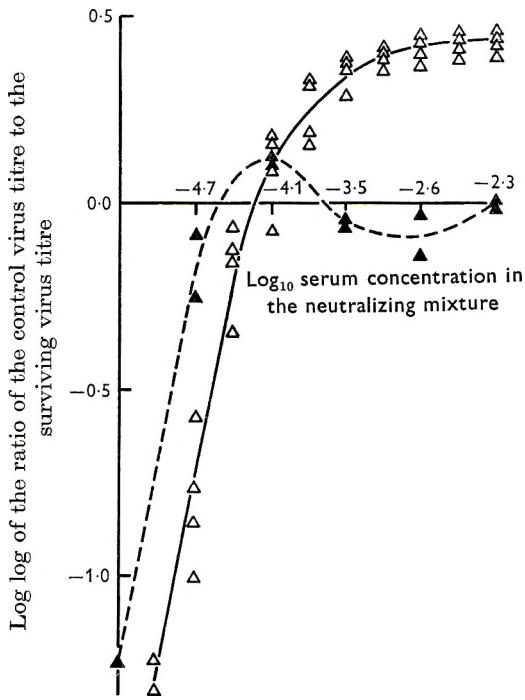

Fig. 6. Log log plot of the neutralization curve of Semliki Forest virus by absorbed hyperimmune rabbit serum. Serum diluent: △, PBS or 0·002 M EDTA in PBS; ▲, PBS containing 1 % (v/v) unheated normal guinea-pig serum.

The effect of unheated normal guinea-pig serum on the neutralization of Semliki Forest virus by antiserum of different origins

We compared the action of unheated normal guinea-pig serum on the neutralization of Semliki Forest virus by: (A) rabbit absorbed antiserum, (B) mouse ascitic fluid, and (C) guinea-pig antiserum (Fig. 5). The two sera and the ascitic fluid had quite different neutralizing potencies, but the shape of the neutralization curves were similar. The effect of unheated normal guinea-pig serum was the same in all instances, decreasing the surviving fraction at low antibody concentrations and increasing it at high concentration of antiserum. The action of unheated normal guinea-pig serum seems independent of the degree of immunization and of the serum donor species, although the extent of the potentiation and of the inhibition is not the same in all three cases.

The log log plot of the neutralization curve

As the neutralization curves obtained with rabbit absorbed antiserum with or without EDTA are identical, we plotted them together (Fig. 6). Log log v_0/v_s was taken as ordinate. v_0 is the virus concentration in the control, v_s the virus concentration in the virus-serum mixture after incubation. The log of the serum concentration was taken as abscissa (Fazekas de St Groth, 1961). The neutralization curve of Semliki Forest virus by absorbed antiserum in the presence of unheated normal guinea-pig serum was plotted in the same way.

DISCUSSION

Studying the neutralization of Semliki Forest virus by rabbit antiserum, we observed that the presence of unheated normal guinea-pig serum slightly potentiated the neutralization when low serum concentrations were used but inhibited the neutralization for greater serum concentrations. The phenomenon was observed with two batches of pooled unheated guinea-pig serum. Heat-inactivated guinea-pig serum had no effect. Addition of EDTA significantly reduced the effect of unheated guinea-pig serum. Thus, the effect of unheated guinea-pig serum on the neutralization appears to be an effect of complement.

When crude virus suspension was used instead of purified virus in the neutralization test, the effect of complement was lessened. Complement could be blocked by the mouse-brain lipids or other substances.

The effect of complement was observed when Semliki Forest virus was neutralized by means of rabbit antiserum, mouse ascitic fluid or guinea-pig antiserum; it is thus not related to the species of the serum donor or to the degree of immunization. Moreover, as on the one hand the effect of complement was more marked when absorbed rabbit serum was used, and on the other hand anti-mouse brain rabbit serum had no neutralizing potency either with or without complement, anti-mouse brain antibodies apparently are not involved in the observed phenomenon. Preimmunization rabbit serum had no neutralizing potency either with or without complement. Complement alone did not modify the virus titre. It thus

appears that the observed phenomenon requires the presence of specific antiviral antibodies as well as the presence of complement; some neutralizing antibodies probably react differently in the presence of complement.

'Complement-requiring neutralizing antibodies' which are potentiated by complement have been described by Yoshino & Taniguchi (1965*a*) working with herpes simplex virus. These antibodies are present in greater proportion in the serum of animals which are bled early after infection than in the serum of hyperimmune animals. Such antibodies are probably also present in the antisera described here, because the potentiation of neutralization by complement at low serum concentration was more marked when immune guinea-pig serum was used instead of hyperimmune rabbit serum. But, in addition, we observed that neutralization by high concentrations of serum was inhibited by complement. This inhibition of neutralization, which is more marked when late serum is used instead of early serum, is probably related to another kind of antibody. These antibodies, unlike the complement-requiring neutralizing antibodies of Yoshino & Taniguchi (1965*a*), would be present in greater proportion in late serum than in early serum. A similar inhibition of neutralization was perhaps observed by Dozois, Wagner, Chemera & Andrew (1949), who noticed that the potentiation of the neutralization of WEE virus in the presence of complement disappeared when the serum concentration in the neutralization mixture was raised to 25 % (v/v).

When the neutralization is plotted on the log log scale, the resulting curve is very similar to the theoretical and experimental curves described by Fazekas de St Groth (1961). The theoretical curve is based on a theory assuming that primary neutralization is a state of equilibrium between association and dissociation of virus–antibody complexes. As our results fit well with this curve, they do not contradict the theory. Moreover, as the values of the surviving fraction obtained with high serum concentrations are lower when undiluted virus–serum mixtures are inoculated to the cells, instead of dilutions of the neutralization mixtures (unpublished results), we have experimental evidence that dissociation does occur. In our tests we measured the surviving fraction of virus after dilution of the neutralizing mixture, so our results were biased towards the measurement of secondary irreversible neutralization. We measured a compound of secondary irreversible neutralization and of the equilibrium neutralization to be found in the final dilution of the virus serum mixture. Because our infectivity assay was always carried out with dilutions of the neutralizing mixture, as is the case in studies of the kinetics of the neutralization, the minimum surviving fraction obtained with high serum concentrations corresponds to the non-neutralizable fraction of Dulbecco *et al.* (1965). According to Fazekas de St Groth & Reid (1958), Fazekas de St Groth (1962) and Lafferty (1963*a*, *b*, *c*) this non-neutralizable fraction is the result, first, of the presence in the antiserum of non-neutralizing antibodies, secondly, of the decreasing probability of irreversible virus–antibody unions as the virus surface becomes saturated with antibodies and, finally, of the increasing importance of non-avid antibodies when the serum concentration is raised. The modification of the neutralization curve in the presence of complement is in good agreement with these hypotheses. The observed potentiation of neutralization would be an

increase of the secondary irreversible binding of avid neutralizing antibodies. The inhibition of neutralization which is, on account of the experimental design, an inhibition of irreversible neutralization, would be either an increase of neutralization by non-avid antibodies (followed upon dilution by dissociation) or less probably an increase of irreversible binding of non-neutralizing avid antibodies. The theory of Yoshino & Taniguchi (1965*b*) assuming that antibodies are always irreversibly attached to the virus but that the neutralization is a secondary process which can be enhanced by complement, could explain the potentiation but could not easily explain the inhibition of neutralization by complement. The lower inhibition of neutralization observed with high concentration of complement could be due to the fact that at the final dilution, when the diluted neutralizing mixture is in contact with the cells, a decrease of the irreversible neutralization is masked by an increase of an equilibrium neutralization involving a joint action of complement and antibody. This is compatible with the fact that this phenomenon was observed at high concentration of serum as well as of complement.

SUMMARY

The neutralization of a constant dose of Semliki Forest virus by various doses of different antisera was studied. The presence of complement (1/200, v/v) in the neutralization mixture inhibited the neutralization by high concentration of antiserum and somewhat potentiated neutralization by low serum concentration. Because the experiments were biased towards a measure of irreversible neutralization, the inhibition observed in the presence of complement appeared to be an inhibition of irreversible neutralization. This inhibition was interpreted as a dissociation of a complement binding virus–antibody complex. The antibodies involved appeared to be virus-specific.

We are indebted for their technical aid to Mrs M. Heusden, Mr R. Mommens and Mr J. L. Renson.

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Note added in proof. Similar findings on the inhibition of virus neutralization were recently described by Westaway, A. G. (1965). The neutralization of arboviruses. I. Neutralization in homologous virus-serum mixtures. *Virology*, **26**, 517.

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A simple fumigation method for disinfecting clothing or bedding containing body lice

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INTRODUCTION

The use of contact insecticides for de-lousing

The public health importance of body lice depends on their prevalence, and so does the most satisfactory method of controlling them. Under conditions of widespread lousiness (either in primative communities or as a result of war or disaster) there is always a threat of louse-borne disease. The best method of preventing such a calamity, or of quenching an actual epidemic, is by rapid elimination of the vector. Application of DDT powder by dusting guns applied to openings in the clothing has proved ideal for this purpose, as in the Naples typhus epidemic (1943), in Korea (1951) and elsewhere.

The phenomenal success of DDT and other synthetic contact insecticides for coping with widespread lousiness has diverted attention from the less important matter of dealing with small but persistent numbers of infested people in civilized conditions. In Britain, these people are usually treated at local authority cleansing stations, at Public Assistance Board Hostels or on admission to H.M. Prisons. Since they are going into a clean environment, protection from reinfestation is not required nor is there need to treat large numbers quickly. On the other hand, the method should destroy eggs as well as lice on the clothing. For these reasons DDT and similar insecticides are not appropriate. Their action is somewhat slow and depends for ovicidal action on the continued wearing of treated underwear. Furthermore, heavy dusting with insecticide powder would be resented by infested persons, who would make extra efforts to avoid treatment. Light applications (e.g. by aerosols) cannot be relied upon and may lead to the development of resistance. We have, in fact, unpublished evidence of the existence of insecticide-resistant body lice in England which is recorded in the following paragraph.

Evidence of insecticide-resistant lice in England

In 1961 a colony of lice was started from an infected man in Stepney, London. The lice were needed to cross with a resistant strain in a genetical investigation (later published by Guneidy & Busvine, 1964). Preliminary tests by the method of Busvine & Lien (1961) revealed that the Stepney strain showed definite signs of resistance to both DDT and dieldrin and it was discarded (Table 1).

Table 1. *Results (% kill) of tests on louse strains*

Insecticide...	Dieldrin								DDT		
Concentration (%)...	0·01	0·025	0·05	0·1	0·2	0·4	0·8	1·6	1	2	4
Stepney	—	—	—	—	29	70	77	100	—	0	0
Cairo	5	48	97	—	—	—	—	—	38	68	—
Orlando	—	—	—	100	—	—	—	—	—	50	89

(The Cairo and Orlando strains are probably normally susceptible. Method, and data for Cairo strain, from Busvine & Lien (1961).)

Heat as an alternative de-lousing method

Many authorities rely on heat-disinfestation, often by rather old-fashioned steam sterilizers. This procedure is rather cumbrous and troublesome; and since most disinfestors are large, it is wasteful to employ them for the garments of only one or two infested people. Furthermore, the temperatures reached may be harmful to some articles of clothing unless care is exercised.

Fumigation for de-lousing

Fumigation has been used for many years to destroy lice in clothing. It can be perfectly satisfactory provided that protection from reinfestation is not required. The more volatile or gaseous fumigants are difficult to handle on a small scale (Busvine, 1943). It is possible to overcome this difficulty; for example, by using small ampoules of methyl bromide to be broken inside a gas-proof bag (Latta & Yeomans, 1943) or by using aluminium-phosphide tablets ('Phostoxin'). We considered both these methods, but there are certain technical drawbacks, apart from the toxicity of these gases, which require the inconvenience of airing out of doors.

In contrast to the wide variety of modern contact insecticides, scarcely any new fumigants have come into use in the past 20 years. We made some trials of the 'residual fumigant' dichlorvos which did not prove promising. Accordingly, it was decided to reconsider the range of liquid fumigants examined by David (1944), who described simple methods of de-lousing by six different compounds. A person seeking guidance on the most satisfactory of these fumigants may be somewhat perplexed and it seemed worth choosing one of them and making confirmatory tests. On balance, ethyl formate seems to combine the desirable properties of safety and convenience with fair potency at both high and low temperatures. Apparently this compound has been used successfully for de-lousing intakes at H.M. prisons for some years.

MATERIALS AND METHODS

The lice used in these experiments came from a normally susceptible colony obtained from the Orlando laboratories of the U.S.D.A. Entomology Research Division. We maintained them in mesh-covered tins, worn daily on the ankle, as described by Buxton (1947).

Lice, or their eggs, were exposed to very low concentrations of dichlorvos

vapour in the simple apparatus described by Khattat & Busvine (1965). Various concentrations of ethyl formate for treatment of lice (or eggs) were prepared in 5 l. flasks. Appropriate volumes of liquid (0·05–1·4 ml.) were pipetted on to glass filter papers suspended in the flasks.

Some practical trials were done in which lice or eggs, in mesh-fronted metal tins, were secreted in four folded blankets. The blankets were then stacked, either in a small metal bin (about 2·8 cu.ft.) with a tight-fitting lid, or in plastic bags (about 30 × 40 in.). The plastic bag is 'sealed' during fumigation by rolling up the open end as far as possible, the final volume then being little more than that of the four folded blankets. The volume was measured by immersing the bag containing blankets in a drum of water and measuring the volume of water displaced, which was about 1·8 cu.ft.

In some trials of ethyl formate, vapour concentrations were determined by the method outlined by David (1944). The samples were drawn from a point about one-quarter the height of the container, usually from the middle of a folded blanket.

RESULTS

Experiments with dichlorvos

The vapour of dichlorvos is extremely potent against flying insects, such as flies and mosquitoes, which are killed by 30 min. exposure to 0·2 μg./l. (Maddock & Sedlak, 1961; Maddock, Sedlak & Schoof, 1961). Unfortunately, lice and their eggs are much more tolerant, the former requiring 9·5 hr. for knock-down and the latter suffering only about 30 % kill after 24 hr, when exposed to concentrations of this order.

In a practical trial, a Shell 'Vapona' resin vaporizer was included in a plastic bag with four folded blankets among which were tins of louse eggs. No reduction of hatch was observed after 6 hr. exposure at room temperature and only about 60 % reduction after 24 hr.

Dichlorvos was therefore considered unsuitable for this purpose.

Experiments with ethyl formate

Measurement of lethal concentrations

Lice and other eggs were exposed to various concentrations of ethyl formate at 10° C. for periods of 1, 2, 5 or 10 hr. In a preliminary test, the gas concentrations attained were determined by samples taken from a flask at the beginning and end of a 5 hr. exposure. They were found to agree very well with expectations based on the dosage.

After exposure, lice were put in tins worn against the skin and examined next day for mortality. The eggs were put into an incubator at 27° C. and 80 % R.H. and examined about 2 weeks later. The kills of louse eggs were corrected for control mortalities, nearly always assessed from an untreated portion of the same batch. These results are given in Table 2.

These data were plotted on logarithmic probability paper and the median lethal concentrations were estimated graphically. Except at the 10 hr. exposure, the

eggs were consistently more tolerant than adult lice, the LC 50 values for the latter being about 80–90 % of the values for eggs. Even greater differences will be found for the lowest lethal concentrations in Table 2, probably because more eggs were used per batch. A more precise statistical treatment would probably be unrewarding, because of disparate defects in the data. Thus, numbers of lice were rather low (average thirteen per batch), and egg numbers, though satisfactory (average forty-seven per batch), were subject to a high, variable, control mortality (average 32 %).

In brief, it appears that eggs are slightly more tolerant of ethyl formate than adult lice.

Table 2. *Results of flask fumigation tests, with ethyl formate, at* 10° C

Concentration (mg./l.)	% kill of lice				Corrected % kill of eggs			
	1 hr.	2 hr.	5 hr.	10 hr.	1 hr.	2 hr.	5 hr.	10 hr.
13	—	—	0	14	—	—	23	—
17	—	—	10	33	—	—	—	50
20	—	—	—	75	—	—	27	64
24	—	—	—	100	—	—	—	100
25·5	—	—	70	—	—	—	55	100
34	—	23	100	—	—	0	90	—
51	—	63	—	—	—	52	100	—
68	—	100	—	—	—	66	—	—
85	—	—	—	—	10	100	—	—
102	40	—	—	—	46	100	—	—
119	66	—	—	—	58	—	—	—
135	100	—	—	—	68	—	—	—
204	—	—	—	—	91	—	—	—
238	—	—	—	—	100	—	—	—
LC 50	108	44	20	17	115	56	24	17

Semi-practical trials

In the earlier work mentioned previously (Busvine, 1943; David, 1944) ordinary metal dust-bins were used for de-lousing fumigations. While this method may be found adequate, we thought that plastic sacks might be more convenient in some ways. Accordingly, we have tried using various plastic bags (about 30 × 40 in.) which are simply sealed during fumigation by rolling up the mouth of the bag. Some experimental fumigations were made with these bags in a cold room at 10° C.; the results may be compared with the recommendations for bin fumigation given by David (1944). In addition, gas samples were taken to estimate concentrations in the bags and in a metal bin under comparable conditions.

Plastic-bag fumigation tests. Batches of lice (average thirty-seven) and eggs (average 188) were divided into four lots, put into mesh-topped tins and secreted in the folds of four army-type grey blankets. These blankets weighed about 4 lb. each; they were folded four times and fumigated in a pile. The fumigant was applied by one of two methods: (A) by dividing the dose into three lots and sprinkling between the blankets; or (B) by sprinkling the full dose on the top blanket.

Table 3. *Results (% kill) of fumigation trials with ethyl formate in plastic bags, at 10° C*

(Method A: fumigant applied in three portions between blankets. Method B: fumigant applied to top blanket.)

		Dose/cu.ft.							
		3 oz. (90 c.c)	2 oz. (60 c.c.)		1 oz. (30 c.c.)		½ oz. (15 c.c.)		¼ oz. (7·5 c.c)
Method...		B	A	B	A	B	A	B	A
1 hr. exposure	Lice	100	100	68	82	—	—	—	—
	Eggs	88	100	56	62	—	—	—	—
5 hr. exposure	Lice	—	—	—	—	100	100	100	100
	Eggs	—	—	—	—	100	100	98	92

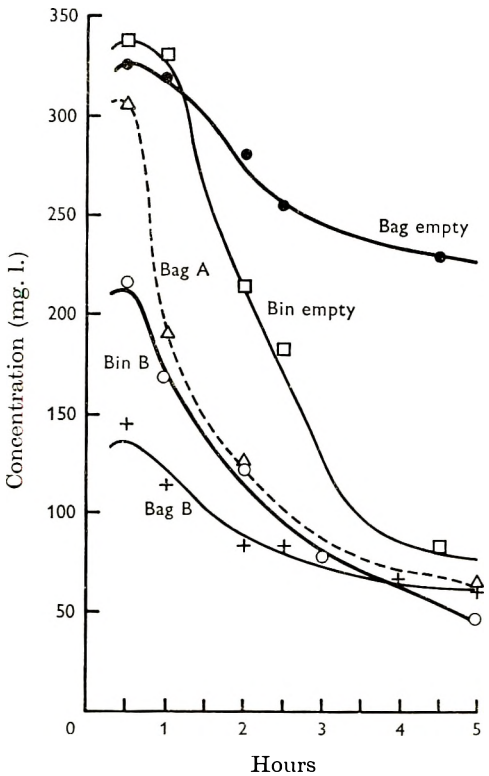

Fig. 1. Concentrations of ethyl formate estimated from samples drawn from a metal bin or a plastic bag. A and B refer to method of applying the dose on a load of blankets. In A it is applied in three portions, between the blankets; in B as a single dose on the top. In all cases the full dose was ½ oz./cu.ft. (corresponding to 500 mg./l.)

The results of trials with 1 or 5 hr. exposure at 10° C. are given in Table 3. It will be seen that method A (as recommended by David, 1944) was more efficient, though it is slightly more troublesome.

Vapour concentration measurements. The concentrations of ethyl formate attained by a dosage of ½ oz./cu.ft. in plastic bag or metal-bin fumigations are

shown by the curves in Fig. 1. These curves are based on results of two or three trials each. The results were reasonably consistent when the dose was applied by method B, the coefficient of error for various points being 11·6 %. When method A was used, the estimation of the initial concentration (after ½ hr.) was very variable, depending on whether some of the liquid applied was close to the sampling point in the blanket fold; but by 2 hr. the levels in three different tests were much the same.

The following points emerge from consideration of these results.

(i) In experiments without blankets the bag volume was maintained by a wire frame. The dose was applied to a cloth suspended at the top of bag or bin. It will be seen that the initial concentration in bag and bin was about the same; but the concentration fell much more sharply in the bin, probably due to leakage.

(ii) When four blankets were present, and the dose was applied by method B, the initial concentration at the sampling point in the blankets was higher in bin than bag. This could have been owing to the smaller total dose in the bag, where the same load as in the bin was packed more tightly in a smaller volume. By the end of the 5 hr. fumigation test, the more rapid loss from the bin had brought down concentrations to that of the bag or below it.

Very similar curves for bag and bin were obtained with a dosage of 1 oz./cu.ft.; but naturally these were at about double the concentration levels attained with ½ oz./cu.ft. (They are not shown in Fig. 1.)

(iii) When the dose of ½ oz./cu.ft. was applied by method A, the initial concentration in the blankets was considerably higher than after application by method B. This was almost certainly due to the fact that with 'A' a portion of the fumigant dose was applied close to the sampling point. After a few hours, however, the concentrations produced by the two methods were about equal, presumably as a result of diffusion.

Conclusions

Our practical trials were all done at 10° C., which was assumed to be the lowest probable room temperature and consequently represented most unfavourable conditions. The results of plastic-bag fumigation tests under these conditions, confirmed the dose recommended by David (1944) for a 5 hr. exposure; but we found recoveries of both lice and eggs with his suggested 30 ml./cu.ft. dose for a 1 hr. exposure. The results of the sampling tests suggest that this may be due to a relatively lower efficiency of the bag method (as compared to a bin) *for short exposures.*

RECOMMENDATIONS

All the work confirms our good opinion of ethyl formate, one of the compounds advocated by David (1944). In addition to its use in metal bins, it can be conveniently used to disinfest clothing or bedding in plastic bags. Under the most unfavourable conditions likely to be encountered indoors, a dose of 2 oz./cu.ft. should be used for a short (1 hr.) exposure; but for 5 hr. or longer ½ oz./cu.ft. is adequate. It is suggested that an overnight treatment is convenient. Using plastic

bags about the size of potato sacks, four army blankets can be disinfested by 25 ml. ($\frac{1}{2}$ oz./cu.ft.) costing about 1*s*. The time to load and treat blankets (or garments) is a few minutes. On their removal there is only a mild smell of the fumigant, which dissipates in a few minutes.

Ethyl formate has the formula $HCOOC_2H_5$; M.W. 74; B.P. 54° C. (135° F.). At 25° C. (77° F.) the density of the vapour is 1·52 times that of air. At 20° C. (68° F.) its vapour pressure is 95 mm. Hg., corresponding to a saturation concentration of 837 mg./l. According to Browning (1953) a concentration of 32 mg./l. is lethal to cats after $1\frac{1}{2}$ hr. and dogs after 4 hr. Its toxicity is not greatly different from that of, say, benzene. It appears that 'no severe effects from the industrial use of ethyl formate have been recorded' (though apparently it is extensively used, at least in the shoe industry). Ethyl formate is inflammable, but in the quantities recommended the danger is negligible.

SUMMARY

Modern contact insecticides (like DDT) in powder form are ideal for combating widespread lousiness; but for various reasons they are not suitable for disinfecting small numbers of infested people under civilized conditions. It appears that the most convenient, efficient method in these circumstances is a small-scale fumigation of the infested garments, in either a metal bin or a plastic bag. Ethyl formate, one of the liquid fumigants suggested for this purpose by W. A. L. David in 1944, has been further tested and found efficient. In a plastic bag the size of a potato sack (about 30 × 40 in.), four blankets can be deloused by a dose of 100 ml. with an hour's exposure or a quarter of this dose with an exposure of 5 hr. or more. An overnight treatment with this dose would be safe and convenient, costing about 1*s*. and the actual operations would take only a few minutes.

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Virological and epidemiological studies on an outbreak of aseptic meningitis caused by echovirus 4 in northern Japan in 1964*

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During the summer and autumn of 1964 a number of localized outbreaks of aseptic meningitis occurred in Japan. The main causative agent of most outbreaks was determined as echovirus 4 by virus isolation in regional laboratories. Among these outbreaks, that through northern Japan seemed to be on the largest scale.

The present communication describes virological and epidemiological studies on the epidemic of aseptic meningitis caused by echovirus 4 in northern Japan.

MATERIALS AND METHODS

Subjects and specimens

Patients with aseptic meningitis examined clinically in 1964 by pediatricians in the medical institutions of eight different regions in northern Japan were studied (see Fig. 1). For virus isolation, cerebrospinal fluids (CSF) and faeces were collected from the patients in the acute stage. Serum specimens were taken from the patients in acute and convalescent phases for serological studies. In addition, faecal specimens were obtained from healthy children in Aomori City every month in 1964 for the examination of seeding of enteroviruses. For sero-epidemiological studies, serum specimens were also collected from children and adults residing in Aomori City. Two groups of serum specimens were obtained from May 1963 to March 1964 and from January to April 1965. These two are referred to as the sera before and after the epidemic, respectively. All these specimens were stored at $-25°$ C. until use.

Virus isolation and identification

Primary cultures of cynomolgous monkey kidney cells were used throughout for virus isolation and identification. Procedures were described in a previous paper (Hinuma, Murai & Nakao, 1965). In this study, however, the medium of the cell cultures inoculated with specimens was replaced by fresh medium every 3 or 4 days and the cultures were inspected for 2 weeks.

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Neutralizing antibody test

Acute and convalescent serum specimens of one patient were always tested on the same day. Sera were inactivated at 50° C. for 30 min. before the test. Fourfold serial dilutions of serum were made and tested against approximately 100 TCD_{50} of test virus. Serum dilution–virus mixtures were allowed to stand at room temperature for 60 min. and 0·2 ml. of these mixtures was inoculated into stationary cell culture tubes. Antibody titres were expressed as the reciprocals of the highest

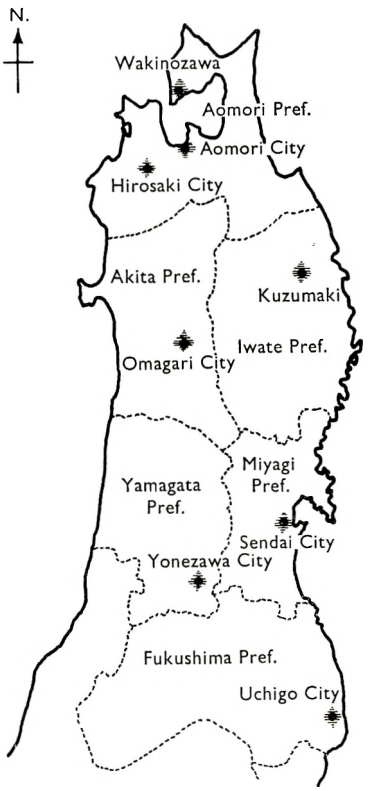

Fig. 1. Regions in northern Japan where outbreaks of aseptic meningitis occurred.

serum dilutions that protected the cells completely after incubation at 36–37° C. for 5–7 days. For the purpose of serodiagnosis, the DuToit strain (Barron & Karzon, 1961) of echovirus 4 was used, unless otherwise stated. For the antibody titration, primary cell cultures of cynomolgous monkey kidney were mainly used, but a line of continuous culture of African green-monkey kidney cells, VERO, established by Dr Y. Yasumura, Department of Bacteriology, Chiba University School of Medicine, was also used in part. The culture of VERO cells was sensitive to the DuToit virus as will be described in the text.

RESULTS

Incidence of patients with aseptic meningitis

The monthly incidence of aseptic meningitis in six regions is shown in Table 1; reliable data from the other two regions were not available. The peak incidence in each region was observed either in July, August or September. In Aomori City, patients with aseptic meningitis associated with echovirus 4 were found from April to December, as will be shown later. In all the regions examined, the peak inci-

Table 1. *Monthly incidence of aseptic meningitis in 6 regions of northern Japan in* 1964

	No. of patients in the following regions:					
	Wakinozawa	Aomori City	Hirosaki City	Kuzumaki	Yonezawa City	Uchigo City
January	0	2	0	0	1	1
February	0	2	0	0	0	0
March	0	5	0	0	1	2
April	0	1	0	0	1	1
May	0	3	10	2	9	2
June	0	12	1	7	9	1
July	45	44	15	30	17	0
August	6	69	47	7	54	14
September	0	48	66	0	22	38
October	0	10	14	0	4	25
November	0	5	0	0	2	9
December	6	7	0	0	2	1
Total	51	208	153	46	122	94

Table 2. *Age and sex distribution of patients with aseptic meningitis in Aomori City and Hirosaki City*

	No. of patients					
	Aomori City			Hirosaki City		
Age (years)	Male	Female	Total	Male	Female	Total
< 1	12	6	18	12	7	19
1	13	6	19	6	7	13
2	14	10	24	9	5	14
3	14	11	25	16	9	25
4	9	13	22	7	4	11
5	13	4	17	13	2	15
6	13	7	20	8	9	17
7	9	4	13	11	5	16
8	11	3	14	8	1	9
9	7	5	12	4	0	4
10	6	5	11	6	2	8
11	4	2	6	0	0	0
12	1	0	1	0	0	0
13	3	1	4	1	0	1
14	2	0	2	1	0	1
Total	131	77	208	102	51	153

dence coincided with the peak of echovirus 4 isolations. The age and sex distribution in Aomori City and Hirosaki City is shown in Table 2. In these two regions about 95 % of cases were infants or children under 10 years; this suggests a poor immunity against echovirus 4 in this age group. The incidence in males was about twice that in females; this seemed to be a characteristic feature of the epidemic due to echovirus 4, in comparison with that in the epidemic of coxsackievirus B5 in Aomori City in 1961 (Nakao *et al.* 1964).

Outline of clinical features

Outstanding symptoms, signs and clinical laboratory findings of the patients in the epidemic were similar to those in many other enterovirus infections.

During the epidemic, one patient with paralysis was observed; this was a 14-year-old boy in Hirosaki City who suffered from flaccid paralysis of upper and lower left extremities on 8 August. Echovirus 4 was recovered from both CSF and faeces obtained 8 days after the onset. The neutralizing antibody titre against the DuToit strain was 16 on both the 8th and the 41st day of disease. No significant rise of antibody titres against three types of poliovirus was found in the paired sera. The results suggest that the paralysis might be associated with echovirus 4 infection.

Among about 800 cases of aseptic meningitis clinically examined in eight regions of northern Japan, nine cases were accompanied by a rash similar to that of rubella. From the faeces of three of them, echovirus 4 was isolated. In the same summer, three strains of echovirus 4, 1 of echovirus 18, 1 of echovirus 25 and 1 of adenovirus were recovered from the faeces of 6 out of 21 patients with rash but without aseptic meningitis. The results may suggest possible aetiological association of echovirus 4 with the rash, although not conclusive.

Virus isolation from the patients

As shown in Table 3, virus was isolated from the CSF of 85 out of 328 patients examined, and 79 (93 %) of the strains were echovirus 4. Viruses were also isolated from the faeces of 152 out of 303 cases. One hundred and thirty-one (86 %) of the strains were identified as echovirus 4. Thus a very large proportion of the strains were found to be echovirus 4, strongly suggesting that this was the main causative agent of the outbreaks in all eight regions.

The monthly incidence of aseptic meningitis patients from whom viruses were recovered either from CSF or faeces or both in Aomori City is shown in Table 4. It is evident that aseptic meningitis associated with echovirus 4 was found from April to December with a high incidence in July, August and September. The incidence of aseptic meningitis cases associated with other enteroviruses was also concentrated in summer, but the total number of such cases was small.

Properties of echovirus 4 isolated in the epidemic

Strains of echovirus 4 isolated in the epidemic examined so far and the Pesascek strain showed cytopathic effect on the primary culture of cynomolgous monkey kidney cells but not on VERO cells, whereas the DuToit strain was cytopathogenic

to both cell cultures. However, all of them, including the Pesascek and DuToit strains, were alike in that they did not show any cytopathic effects on HeLa and FL cells, and were not pathogenic for unweaned mice by intraperitoneal and intracerebral inoculations. The large round plaques of the current strains formed on monkey kidney cells were very similar to those of the Pesascek strain but not to the minute plaques of the DuToit strain, as previously reported by Barron & Karzon (1961).

Table 3. *Virus isolation from the patients with aseptic meningitis*

CSF

Region	No. of specimens tested	No. of virus isolated: Coxs A9	Coxs B3	Echo 4	Echo 6	Echo 25	U*	Total
Wakinozawa	40	—	—	7	—	—	—	7
Aomori City	176	—	1	46	1	1	1	50
Hirosaki City	17	—	—	5	—	—	—	5
Kuzumaki	19	—	—	8	—	—	—	8
Omagari City	0	—	—	—	—	—	—	0
Sendai City	13	—	—	2	—	—	—	2
Yonezawa City	14	—	—	3	—	—	—	3
Uchigo City	49	2	—	8	—	—	—	10
Total	328	2	1	79	1	1	1	85

Faeces

Region	No. of specimens tested	No. of virus isolated: Polio 3	Coxs B3	Echo 1	Echo 4	Echo 6	Echo 25	U*	Total	No. of patients: Tested	Virus-positive†
Wakinozawa	44	—	—	—	17	—	—	—	17	51	19
Aomori City	188	1	3	3	81	3	4	6	101	202	119
Hirosaki City	11	—	—	—	5	—	—	—	5	17	7
Kuzumaki	18	—	—	—	9	—	—	—	9	23	12
Omagari City	10	—	—	—	8	—	—	—	8	10	8
Sendai City	12	—	—	—	3	—	—	1	4	10	4
Yonezawa City	13	—	—	—	7	—	—	—	7	16	8
Uchigo City	7	—	—	—	1	—	—	—	1	50	12
Total	303	1	3	3	131	3	4	7	152	379	189

* U = unidentified.

† Positive isolation of virus in either CSF or faeces or both.

In order to examine the sensitivity of the current echovirus 4 to human antibody, titres of neutralizing antibodies in the paired sera of seven patients with aseptic meningitis were compared by using KZ-3-64 and W-45-64 strains, selected as representative of the current strains, together with the Pesascek and DuToit strains. The conventional tube-neutralization test with monkey kidney cells was performed. A marked breakthrough phenomenon (Barron & Karzon, 1961) was

noticed in tubes with KZ-3-64 and W-45-64 as well as those with Pesascek, but not in those with DuToit. Titres of neutralizing antibody are shown in Table 5.

Low titres were found in all the paired sera by using either of the two current strains or Pesascek, whereas high titres were detected by DuToit so that a significant rise of antibody titre between paired sera was easily demonstrated. The results indicated that the current strains were similar to the Pesascek but not to

Table 4. *Monthly incidence of virus-positive aseptic meningitis in Aomori City*

	No. of patients tested	No. of patients from whom the following viruses were isolated							
		Polio 3	Coxs B3	Echo 1	Echo 4	Echo 6	Echo 25	Unidentified	Total
January	2	—	—	—	—	—	—	—	0
February	2	—	—	—	—	—	—	—	0
March	5	—	—	—	—	—	—	—	0
April	1	—	—	—	1	—	—	—	1
May	3	—	—	—	1	—		—	1
June	12	1	2	—	5	—	—	—	8
July	44	—	1	—	21	2	3	2	29
August	67	—	—	3	34	1	1	—	39
September	46	—	—	—	30	—	—	3	33
October	10	—	—	—	6	—	—	—	6
November	5	—	—	—	1	—	—	—	1
December	5	—	—	—	1	—	—	—	1
Total	202	1	3	3	100	3	4	5	119

Table 5. *Titres of neutralizing antibody tested against 4 strains of echovirus 4 in paired sera of the patients*

Patient no.	D.D.†	Titre of antibody against the following strains* KZ-3-65	W-45-65	Pesascek	DuToit
KZ-3-65	4	< 4	ND‡	< 4	< 4
	23	16		16	256
KZ-12-65	2	< 4	ND	< 4	4
	17	4		4	16
KZ-13-65	2	< 4	ND	< 4	4
	18	16		16	64
W-1-65	1	ND	< 4	ND	4
	17		4		64
W-11-65	1	ND	< 4	ND	4
	15		4		64
W-15-65	6	ND	< 4	ND	< 4
	20		4		64
W-17-65	3	ND	< 4	ND	< 4
	17		16		64

* About 100 TCD_{50}/0·1 ml. of each virus strain was used.
† Days after onset of illness.
‡ ND, Not done.

the DuToit strain in their sensitivity to human antibody. It was also shown that about 100 TCD_{50}/0·1 ml. of the strains of echovirus 4 isolated were neutralized by four units of monkey antiserum against Pesascek strain in the conventional tube method.

Neutralizing antibody against echovirus 4 *in the patients*

Table 6 shows the results of neutralizing antibody estimations against the DuToit strain in paired sera from sixty-one patients with aseptic meningitis. A fourfold or greater rise in antibody titre between paired sera, which was considered as significant, was demonstrated in thirty-two (86 %) of thirty-seven

Table 6. *Neutralizing antibody against echovirus* 4 *in the paired sera of patients with aseptic meningitis*

	No. of patients	Antibody present		Antibody absent
		Rise	No rise	
Patients with positive isolation of echovirus 4 from CSF				
Wakinozawa	4	4	0	0
Aomori City	1	1	0	0
Hirosaki City	5	3	2	0
Kuzumaki	5	4	1	0
Omagari City	0	0	0	0
Yonezawa City	2	1	1	0
Total	17	13	4	0
Patients with positive isolation of echovirus 4 from faeces only				
Wakinozawa	8*	8	0	0
Aomori City	2	2	0	0
Hirosaki City	0	0	0	0
Kuzumaki	1	1	0	0
Omagari City	8	7	1	0
Yonezawa City	1	1	0	0
Total	20	19	1	0
Patients from whom no virus was isolated				
Wakinozawa	6	6	0	0
Aomori City	2	1	1	0
Hirosaki City	0	0	0	0
Kuzumaki	3	2	0	1
Omagari City	2	1	1	0
Yonezawa City	11	7	4	0
Total	24	17	6	1

* In three patients from whom faeces were not available the virus was isolated from throat swabs.

patients from whom echovirus 4 was recovered either from CSF or faeces. The remaining five (14 %) of the virus-positive patients showed the presence of antibody without a rise in titre between the paired sera. Out of twenty-four patients with aseptic meningitis from whom virus isolation was unsuccessful, seventeen

(71 %) showed a significant rise in antibody titre, 25 % showed antibody without a significant rise in titre, and 4 % showed no detectable antibody titre.

Titres of neutralizing antibody plotted against the days after the onset of illness are shown in Fig. 2. Antibody titre of 4 or lower was found within 6 days after the onset, and a titre of 16 or higher occurred between 10 and 20 days after the onset.

Thus serodiagnostic results, in accordance with results of virus isolation, suggested that almost all the patients with aseptic meningitis were associated with echovirus 4 infection.

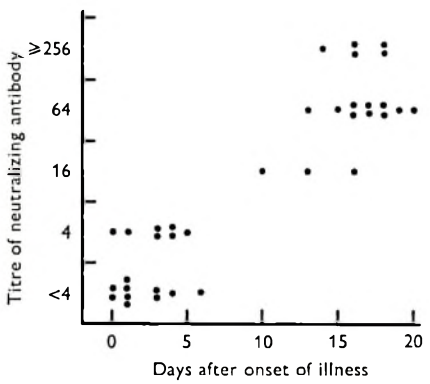

Fig. 2. Titres of neutralizing antibody against echovirus 4 in paired sera of the patients with aseptic meningitis in Wakinozawa.

Table 7. *Enteroviruses isolated from healthy children and infants in Aomori City in* 1964*

	No. of persons tested	No. of persons of positive isolation of the following virus types							
		Coxs		Echo				Unidentified	Total
		A 9	B 3	1	4	14	23		
January	20	—	—	—	—	—	—	1	1
February	128	—	—	—	—	—	—	—	0
March	74	—	—	—	—	—	—	—	0
April	44	—	—	—	—	—	—	—	0
May	67	—	—	—	—	—	—	—	0
June	101	—	1	—	—	—	1	—	2
July	119	—	2	—	1	—	1	—	4
August	103	—	1	2	4	2	—	1	10
September	65	1	—	1	—	1	—	2	5
October	80	—	—	2	—	—	—	2	4
November	16	—	—	—	—	—	—	—	0
December	101	—	1	—	—	—	—	—	1
Total	918	1	5	5	5	3	2	6	27

* Between March and May, 5, 3 and 5 strains of poliovirus type 1, 2 and 3 respectively were isolated. They were considered to be vaccine virus, because Sabin's oral vaccine was given to a number of infants in the city during March and April. These polioviruses are therefore not included in this Table.

Enteroviruses isolated from healthy persons in Aomori City

During 1964 faecal specimens were collected every month from healthy children and infants under 6 years of age in Aomori City, and were tested for the presence of virus using monkey kidney cells. The results are shown in Table 7. Of 918 specimens tested, twenty-seven enteroviruses were isolated, most of them during the summer and fall. Among these viruses, five (19 %) were echovirus 4, and the isolation rate of this virus was the same as that of coxsackievirus B 3 or echovirus 1, viruses which showed very little association with aseptic meningitis in Aomori City in 1964 (see Table 3).

Table 8. *Incidence of neutralizing antibodies against echovirus 4 in Aomori City before and after the 1964 epidemic*

	Before epidemic			After epidemic		
		Positive antibody*			Positive antibody	
Age (years)	No. tested	No.	%	No. tested	No.	%
0–½	15	1	6·7	27	7	25·9
½–1	10	0	0	24	3	12·5
1	12	0	0	23	3	13·0
2	11	1	9·1	25	12	48·0
3	8	0	0	13	2	15·4
4	10	0	0	12	4	33·3
5–6	21	1	4·8	25	4	16·0
7–9	19	1	5·3	24	2	8·3
10–14	22	5	22·7	20	10	50·0
15–19	11	8	72·7	14	11	78·6
20–29	13	6	46·2	15	12	80·0
30–39	10	6	60·0	12	6	50·0
40–49	6	4	66·7	14	10	71·4
⩾ 50	8	5	62·5	16	12	75·0

* Antibody titre of 4 or higher was considered as positive and below 4 negative.

Sero-immunity against echovirus 4 of residents in Aomori City

Serum samples from children and adults in Aomori City, which were obtained before and after the epidemic of aseptic meningitis in 1964, were examined for antibodies against echovirus 4. The sera were from persons free from aseptic meningitis during the past year. The DuToit strain was used for the neutralization test. As seen in Table 8, almost all the children under 10 had no detectable antibody before the epidemic, whereas a much higher proportion of antibody-positive persons was found among children over 10 and adults. This suggested that echovirus 4 had once spread in this region about 10 years ago. After the 1964 epidemic, the proportion of sero-positive persons increased in almost all age groups, suggesting that many persons suffered from an inapparent infection with echovirus 4 during the epidemic. Figure 3 shows actual titres of neutralizing antibody in the sero-positive individuals before and after the epidemic. Before the

epidemic, antibody titres of three children under 10 were only 4 units and those above 10 were between 4 and 64 units. However, much higher titres were found even among children under 10, after the epidemic.

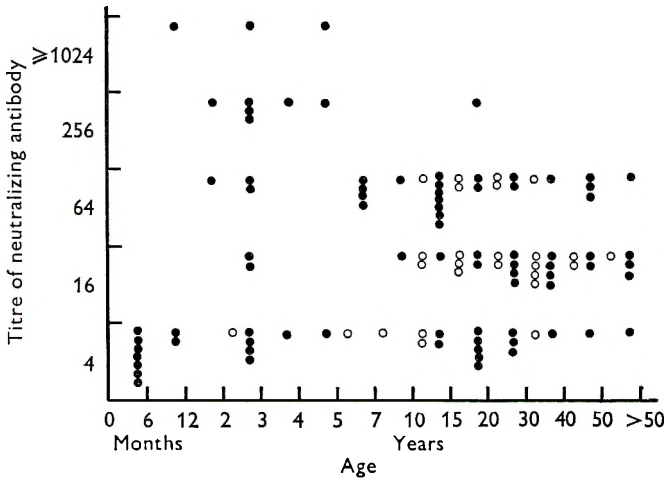

Fig. 3. Titres of neutralizing antibody against echovirus 4 in sera obtained from healthy persons in Aomori City before (○) and after (●) the 1964 epidemic.

DISCUSSION

The main causative virus of the 1964 epidemic of aseptic meningitis which occurred in many regions of Japan, including northern Japan reported here, was determined as echovirus 4 except in one outbreak of aseptic meningitis associated with coxsackievirus B4 in Shizuoka (personal communication from Dr R. Kono, Central Virus Diagnostic Laboratory, National Institute of Health of Japan). Isolation of echovirus 4 in Japan has been described in only one report (Hayakawa, 1964) that the virus was isolated from two sporadic cases of aseptic meningitis in Kyoto in 1963. In northern Japan echovirus 4 had never been isolated before 1964 (Hinuma *et al.* 1964; Hinuma *et al.* 1965). There have been no other reports of epidemics of echovirus 4 in Japan. However, studies of age-specific sero-immunity against echovirus 4 here described strongly suggested that the virus had spread in Aomori about 10 years ago, in 1954. This might be extended to the other regions in Japan, since the range of ages of the patients with echovirus 4 aseptic meningitis in many other regions in 1964 was similarly under 10 years. During 1954–64 there might have been no extensive seeding of the virus, as is suggested by the lack of immunity in the children and infants under 10 in 1964. The 1964 epidemic of echovirus 4 might not have covered most parts of Japan, because several outbreaks of aseptic meningitis due to echovirus 4 have been observed in the summer of 1965 in regions of southern Japan where outbreaks of echovirus 4 were not found in 1964 (unpublished observation). The scale of the epidemic of aseptic meningitis due to echovirus 4 in 1964 and 1965 appeared to be similar to or larger than that caused by coxsackievirus B5 in Japan in 1960 and 1961 (Kono *et al.* 1960; Hinuma *et al.* 1964).

Outbreaks of aseptic meningitis associated with echovirus 4 have been reported from many parts of the world, as listed in Table 9. Thus the accumulated reports emphasize that echovirus 4 is one of a few important viruses in the aetiology of large epidemics of aseptic meningitis.

Table 9. *Epidemics of aseptic meningitis associated with echovirus* 4

Year	Place	Publication
1955	U.S.A.	Levan *et al.* (1957)
1956	U.S.A.	Karzon *et al.* (1961)
1956	Sweden	Johnsson, Böttiger & Löfdahl (1958)
1956	Switzerland	Krech (1957)
1956	Australia	Forbes (1958)
1957	South Africa	Malherbe, Harwin & Smith (1957)
1958	Germany	Munk & Nasemann (1959)
1963	Scotland	Bell (1964)

Echovirus 4 strains isolated in the 1964 epidemic were characteristically insensitive to human antibody: this was similar to the Pesascek strain but unlike the DuToit strain. Tissue-culture spectrum and plaque morphology of the current strains were also similar to those of the Pesascek but different from the DuToit. However, a preliminary examination showed that there was some difference between the antigenic structure of the current strains and the Pesascek (unpublished data).

SUMMARY

During the summer and autumn of 1964, echovirus 4 was found widely distributed in northern Japan; this virus was associated with localized outbreaks of aseptic meningitis in many regions. The evidence that the echovirus was aetiologically related to aseptic meningitis was shown by the virus isolation from cerebrospinal fluids and faeces and the serodiagnosis of paired sera in many patients. Age-specific sero-immunity and morbidity rate suggested that echovirus 4 had spread extensively in about 1954. The strains of echovirus 4 isolated in the 1964 epidemic were similar to the prototype strain, Pesascek, in biological characteristics.

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Distribution of heat-resistant *Clostridium welchii* in a rural area of Australia

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The fact that *Clostridium welchii* occurs universally in nature has been well documented. Smith (1955) claims that *Cl. welchii* is more widely spread over the face of the earth than any other pathogenic bacterium. Taylor & Gordon (1940) reported finding *Cl. welchii* in 190 of 196 soil samples examined, the majority being type A. Hobbs (1962) found that the organism occurs regularly in dust—89·6% of samples containing haemolytic and 81·0% containing non-haemolytic *Cl. welchii*. Lowbury & Lilly (1958) found that *Cl. welchii* is regularly present in the hospital environment, being carried in from outside by dust and in the alimentary canal of patients.

The alimentary canal of humans and animals has long been known to contain *Cl. welchii*. Smith & Crabb (1961) found *Cl. welchii*, often in large numbers, in the alimentary canal of most farm and domestic animals, including dogs, cats, pigs and cows, and also in 80% of humans examined. Collee, Knowlden & Hobbs (1961) and Taylor & Gordon (1940) both isolated *Cl. welchii*, using enrichment techniques in liquid medium, from 100% of humans examined.

The above isolations have all been of the non-heat-resistant, haemolytic *Cl. welchii*, now considered part of the normal flora of man. The investigations by Hobbs *et al.* (1953), revealing heat-resistant non-haemolytic *Cl. welchii* as a cause of food poisoning, began a study by many to determine the carrier rate of this variant in the alimentary canal of man.

Figures for the general population include 2·2% (Hobbs *et al.* 1953), 6–8% (Collee *et al.* 1961), 14% in male workers (Leeming, Pryce & Meynell, 1961) and 20% in healthy hospital personnel and their families (Dische & Elek, 1957). Hospital patients appear to have unusually high carrier rates. Leeming *et al.* (1961) indicate that 30% of hospital patients carry the organism, while Turner & Wong (1961) demonstrated heat-resistant spores in 63% of Chinese hospital patients in Hong Kong—the same workers (using the same techniques) could demonstrate only a 9% incidence of the organism in the general population of Leeds (England). Carrier rates are generally high following an infection—97% at the time of infection (Dische & Elek, 1957) to 50% 2 weeks after the infection (Hobbs *et al.* 1953). The variations in carrier rates expressed by different workers may be due to slightly different techniques, although the results of the examples cited above were all obtained by the use of very similar techniques. It does, however, seem apparent that the carrier rate is now higher in hospitals, boarding schools and other places of communal feeding than in the general population.

In the light of these results it was decided to determine the carrier rate of heat-resistant *Cl. welchii* in a rural area on the North Coast of New South Wales, Australia, and to gauge the effect of age, sex, season and hygiene on this carrier rate.

MATERIALS AND METHODS

The subjects

Faecal specimens were collected in sterile containers from five selected classes of the population.

Class A: primary-school children. Four hundred and sixty-one specimens were examined from non-aboriginal school-children, both male and female, ranging in age from 4 to 11 years. The children were from outlying country areas; the total enrolment at any one school being little more than thirty pupils. They therefore had little association with bulk-prepared canteen-type meals.

Class B: boarding-school students. Fifty-three specimens were examined from male students between the ages of 10 and 18 years. All were taken in mid-term, so that the students had been eating bulk-prepared food for several weeks. The above fifty-three students all attended the same school.

Class C: general public. This class consisted of fifty mildly ill persons attending their own general practitioner, and contained no aboriginal persons. Ages were not available in all cases, but they represent a good distribution of male and female persons from birth to 60 years of age.

Class D: hospital patients. This class consisted of forty-eight patients. A specimen of faeces was collected from each within 6 hr. of entering hospital (class Di) and a second specimen after they had been in hospital for 8 days (class Dii). Results of the two classes have been compiled separately as it was considered that although all specimens were from the same class of patients, they actually represent two distinct classes of persons living under different environmental conditions, particularly with regard to eating habits. The first specimen of faeces represents one from a member of the general public. The second specimen represents one from a member of a hospital environment.

Class E: aboriginals. Four hundred and twenty specimens were examined from aboriginals in four reserves and from a group living within the city, under conditions common to the white population. Two hundred and eighteen of the specimens were collected in the period November—April, and 202 were collected in the winter months of May, June and July.

Collection of material and culture methods

The majority of the faecal specimens were collected in sterile containers from the population classes A–E mentioned above. Samples from 150 primary-school children and 100 aboriginals were collected into clean, non-sterile containers. Numerous cultures on broth washings from such containers showed the absence of *Cl. welchii*, and the low incidence of heat-resistant *Cl. welchii* in primary-school children indicate that these containers introduced little or no *Cl. welchii*.

Where possible the stools were examined within 2 hr. of collection, but in a few

cases, particularly those from an aboriginal reserve in an outlying area, they were not examined until 6–12 hr. after collection.

Media used. The egg-yolk medium was that of Willis & Hobbs (1958), with the medium base adjusted to pH 7·0. Other media used were blood agar (7·5 % defibrinated horse blood added to Oxoid blood-agar base) and Robertson's cooked-meat medium. Neomycin sulphate (Upjohn) was added to the egg-yolk medium and blood agar in a concentration of 100 μg./ml.

All faecal specimens received were examined for heat-resistant *Cl. welchii* by the method now commonly accepted by most bacteriologists (Hobbs *et al.* 1953, Leeming *et al.* 1961, Collee *et al.* 1961). One to two grams of all specimens were inoculated into a tube of cooked-meat medium which was then boiled for 60 min and incubated overnight at 37° C. In this way any heat-resistant spores present were concentrated. It was then subcultured on to egg-yolk medium and blood agar and incubated anaerobically for 24 hr.

On the egg-yolk medium colonies were 1–3 mm. in diameter, with a smooth texture and entire edge. They were cream on first isolation, surrounded by a zone of opalescence due to the lecithinase reaction, and a diffuse red zone due to the fermentation of the lactose in the medium. On standing the colonies themselves acquired a red colour. On blood agar the heat-resistant *Cl. welchii* showed little or no haemolysis. Colonies suspected of being *Cl. welchii* were further examined by gram stain, sugar fermentation tests and for the inhibition of the lecithinase reaction on egg-yolk medium by *Cl. welchii* antitoxin.

Serology

Antisera for Hobbs types 1–13 were kindly supplied by Dr H. Cherry of Communicable Disease Center, Atlanta, Georgia, U.S.A. Using these antisera the organisms classified as *Cl. welchii* were typed by the slide-agglutination technique; the sera were diluted within the range 1/5–1/15 (Hobbs *et al.* 1953).

RESULTS

Distribution of heat-resistant Cl. welchii *in various sections of the population of north eastern N.S.W.*

The results for classes A–E are shown in Table 1.

The figures given in Table 1 indicate that the six different classes of the population can be combined into two distinct larger groups.

Group 1: comprising classes A, C and Di. They all have a low carrier rate of heat-resistant *Cl. welchii* (1·5–6·0 %) and represent the majority of persons in the population.

Group 2: comprising classes B, Dii and E. They all have a high carrier rate (15·1–25 %) of heat-resistant *Cl. welchii*. This group consists of persons associated with either communal feeding or a low standard of hygiene and represents a minority group in the population.

Chi-squared tests performed on these two larger groups indicate that there is no significant difference between classes within the same group (Table 2).

Age distribution of positive specimens

This was only examined in the aboriginal population. In this class a large number of specimens was positive, and the figures therefore afford some comparison of the distribution of heat-resistant *Cl. welchii* among various age groups. The population has been divided into five age divisions (Table 3), each division

Table 1. *Heat-resistant* Clostridium welchii *infection rates of selected classes of the human population of north-eastern New South Wales*

Classes examined	No. specimens examined	Percentage positive
A	461	1·5
B	53	15·1
C	50	6·0
Di	48	4·2
Dii	48	25·0
E	420	19·0

A χ^2 test on these six classes gave a value of $\chi^2 = 79{\cdot}672$.

Table 2. *Some statistical data of results given in Table* 1

Group	χ^2	n	P
1	6·02	3	0·11
2	1·748	3	0·63

Table 3. *Distribution of heat-resistant* Clostridium welchii *among age groups of the aboriginal population*

Age group (years)	No. examined	Percentage positive
0–5	95	6·3
6–10	97	24·7
11–20	85	18·8
21–40	89	18·0
Over 40	54	33·0
Total	420	19·0

containing approximately the same number of persons. The numbers shown are a good representation of the aboriginal population, approximately 66 % being under the age of 21.

The results given in Table 3 were analysed statistically with the following results:

$$\chi^2 = 19{\cdot}424,\ n = 5,\ P = \text{less than } 0{\cdot}01,$$

which indicates that the difference in distribution of heat-resistant *Cl. welchii* among the various age groups is highly significant.

The age groups 0–5 ($t = 4{\cdot}09$, $P = < 0{\cdot}01$) and over 40 years ($t = 2{\cdot}09$, $P = 0{\cdot}94$) differ significantly from the overall aboriginal population.

Distribution of heat-resistant Cl. welchii *among male and female persons*

The results shown in Table 4 indicate that the distribution is fairly even; of 555 males examined 60 (10·8 %) were positive, and of 525 females 52 (9·9 %) were positive. Statistically the figures do not indicate any significant variation in carrier rates between males and females: $t = 0{\cdot}5$, D.F. $= \infty$, $P = 0{\cdot}62$.

Table 4. *Distribution of heat-resistant* Clostridium welchii *among males and females*

	Male		Female	
Group examined	No. examined	No. positive	No. examined	No. positive
A	221	4	240	3
B	53	8	—	—
C	27	2	23	1
Di	21	1	27	1
Dii	21	5	27	7
E	212	40	208	40
Total	555	60	525	52

Seasonal variation

Of the 218 aboriginal samples collected during the summer months 46 (21·0 %) were positive, and 34 (16·8 %) of the 202 examined in the winter months were positive.

Statistically the figures do not indicate a significant difference in the carriage of heat-resistant *Cl. welchii* during the summer and winter months ($t = 1{\cdot}105$, D.F. $= 419$, $P = 0{\cdot}27$).

Serological types of Cl. welchii *isolated*

In order to study the epidemiology and carrier distribution of *Cl. welchii* with any degree of accuracy, all heat-resistant strains isolated, excepting twenty isolated from aboriginals early in the survey, were typed.

Some specimens were found to agglutinate with more than one serum, but in most of such cases one serum gave a stronger, more rapid agglutination than the others. Types 3, 4 and 5 demonstrated this phenomenon while types 9, 11 and 13 in many cases also cross-agglutinated.

No specimens fitted into serological types 4, 5, 7, 8 and 12, so these have been omitted from Table 5, which shows the complete typing results of the six classes. Unfortunately, because of conditions present at the time when this work was done, it was not possible to prepare rabbit antisera against the fifteen untypable specimens.

The carrier state and distribution within family groups

Twenty aboriginals from reserve A, positive in the original sampling, were resampled at 5 and 10 weeks to see whether the carriage of heat-resistant *Cl. welchii* is a transient or permanent state. The results indicate a transitory carriage. No patient carried the same strain in both resamples and in the original sampling.

The figures in Table 6 show that after 5 and 10 weeks only 30 % of the specimens were positive. This is similar to the overall carrier rate of the reserve concerned, indicating that the twenty persons have now reverted to a random sample of the population, and no longer represent twenty selected (positive) persons. In addition, the table shows that almost all persons still positive at the resamples carried a different serotype from that isolated at the original sampling. This is evidence against a permanent carrier state.

Table 5. *Serological classification using Hobbs's typing sera* 1–13, *of heat-resistant* Clostridium welchii *isolated from selected classes of the population*

Hobbs's type	Class of patients					
	A	B	C	Di	Dii	E
1	1	1	—	—	—	6
2	—	—	—	1	—	2
3	—	1	—	—	—	4
6	2	1	1	1	—	12
9	1	—	1	—	4	6
10	2	2	—	—	4	8
11	—	1	—	—	—	3
13	—	1	—	—	3	8
Untypable	1	1	1	—	1	11
Total	7	8	3	2	12	60

Table 6. *Result of specimens resampled* 5 *and* 10 *weeks after original sampling*

Time examined after original sample (weeks)	No. sampled	No. containing heat-resistant *Cl. welchii*	No. carrying same serotype as in original sampling
0	20	20	—
5	20	6	1
10	20	7	—*

* One specimen carried the same serotype as at 5 weeks but this differed from the serotype found in the original sampling.

One woman on entering hospital was a carrier of Hobbs type 6, but after 10 days in hospital this organism had been replaced by Hobbs type 9—present in three other members of her ward at that time. A further specimen, submitted 4 weeks after her hospital discharge, contained no heat-resistant *Cl. welchii*. In the hospital, where the two wards examined were served by the same kitchen, it was evident that one organism was predominantly present at any one time. Inspection of the serological types present in the hospital environment (Dii) shows that only three types were present. The presence of each type corresponded to a period of time, after which it was replaced entirely by a different serotype. The woman discussed above served as a fine example to illustrate this point.

No aboriginal reserve, as a whole, showed the carriage of any one predominant serotype. In cases where members of the same family were carriers of heat-resistant *Cl. welchii* there were many examples of the same serotype being carried

by two or more members of the same family at the one time. In one case, however, three members of a family all carried different serotypes at the one time. There does, therefore, appear to be an association between carriage of heat-resistant *Cl. welchii* and certain families. Usually those families with poor hygienic conditions have several members carrying heat-resistant *Cl. welchii*. The seventy-eight families examined were divided into good, fair and poor hygiene, depending upon the general cleanliness of their dwellings, their persons and their habits. The use of refrigerators and other methods of food storage was also considered important. The distribution of heat-resistant *Cl. welchii* among these families is given in Table 7. A family in this case constitutes a group of persons living together and sharing the same meals.

Table 7. *Distribution of heat-resistant* Clostridium welchii *among aboriginal families with good, fair and poor hygiene*

Living conditions	No. of families examined	No. with one or more member infected	Percentage infected
Good hygiene	20	4	20
Fair hygiene	32	18	60
Poor hygiene	26	24	92

$\chi^2 = 26{\cdot}227$, $n = 3$, $P = < 0{\cdot}01$.

It is obvious from the results that a definite association between poor hygiene and the carriage of heat-resistant *Cl. welchii* among aboriginal families does occur.

Of the 26 families with poor hygiene, 24 had more than one member a carrier. There were 58 isolations of heat-resistant *Cl. welchii* from these 24 families. These results indicate that 72·5% of positive specimens occur in 30·8% of the families investigated. This is evidence for the hypothesis of carriage within family groups—those families concerned having a poor standard of hygiene. This, of course, does not mean that all carriers come from families with poor hygienic conditions. Most carriers within the white population acquire the organism from canteens, where conditions for correct food storage are not adequate, or not fully utilized. The aboriginals on the other hand have little association with canteens, and in most would acquire the organisms from their home environment.

DISCUSSION

The carrier rate recorded for the majority of persons on the north coast of N.S.W. (group 1, comprising classes A, C and Di) is lower than that recorded by many workers (Dische & Elek (1957), 20%; Leeming *et al.* (1961), 14%). This is probably due to the fact that the area is a predominantly rural one, with little industrial activity, and so with very little association with canteen-type food. It is noticeable however that persons associated with communal feeding (hospital patients, and boarding-school students) and with a low standard of hygiene (aboriginals) have a much higher carrier rate (15·1–25%) than the majority of the

population (1·5–6·0 %). These results are in agreement with those of Leeming *et al.* (1961) who demonstrated an increased carrier rate in hospital patients, and with Turner & Wong (1961) who demonstrated 63 % carriage of heat-resistant *Cl. welchii* in Chinese hospital patients of Hong Kong and only 9 % in the general population of Leeds (England). Selection and transmission of heat-resistant strains by bulk cooking followed by slow cooling of the meat could partly account for this predominance of heat-resisant *Cl. welchii* in hospitals and institutions. In the aboriginal persons examined it was evident that age was a significant factor in the distribution of heat-resistant *Cl. welchii* (χ^2 = 19·424). The age groups 0–5 and those over 40 years differ significantly from the mean aboriginal carrier rate (t = 4·09 and 2·09 respectively). This difference in the carriage of heat-resistant *Cl. welchii* is probably related to differences in eating habits of the various age groups. This applies particularly to the 0–5 age groups, where most are breast-fed in infancy, and even up to the age of 3 or 4 years eat specially prepared meals, and do not partake in the normal family meal. Results show that the distribution of the organism among males and females is similar. Leeming *et al.* (1961) were able to demonstrate a higher carrier rate in men, but explained that this was probably due to the eating habits—the men tending to eat a large number of canteen-prepared meals. In hospital patients the male and female carrier rates became the same. There would therefore appear to be no reason (other than eating habits) why male and female persons should not have similar carrier rates of heat-resistant *Cl. welchii*. All this work clearly indicates that the carriage of heat-resistant *Cl. welchii* is closely linked with communal feeding and poor hygienic conditions (χ^2 = 26·227 for hygiene); in both cases the food eaten is often left unrefrigerated for some hours between cooking and the actual consumption. The Ministry of Health (Report, 1964) reports that 60 of 62 outbreaks of food poisoning due to *Cl. welchii* occurred in either hospitals, school canteens, restaurants or institutions.

Particularly interesting was the result indicating that there was no significant difference in the carriage of heat-resistant *Cl. welchii* in summer or winter months. This is not in agreement with the occurrence of food-poisoning due to other organisms. Wilson & Miles (1955) point out that cases of bacterial food-poisoning are most frequent in the summer months. In England and Wales during 1963 (Report, 1964) 47 % of *Salmonella* food-poisoning outbreaks occurred during the months June to August, and all staphylococcal outbreaks occurred from March to October. On the other hand, the same table of results indicates that outbreaks of *Cl. welchii* food-poisoning occurred regularly throughout the year.

This may be due to the fact that in most cases of *Cl. welchii* food-poisoning the meat dish is naturally contaminated before it reaches the kitchen. The food handler plays little part in the contamination of the food. The heat-resistant spores are able to withstand the cooking process, particularly if the portion of meat being cooked is large and bulky. As the meat dish cools, the temperature of the meat drops to 50° C. within 2½ hr. but then cools more slowly, remaining within the temperature range of 20–50° C. for many hours (McKillop, 1959). This is particularly so in a warm kitchen, where several roasts are allowed to stand together

after cooking. This offers ideal opportunities for the heat-resistant spores, already present, to germinate and multiply. Collee *et al.* (1961) were able to demonstrate that heat-resistant *Cl. welchii* grows rapidly within a temperature range of 23–50° C. In the cold winter months, if the meat be drained of its broth and put aside from other portions of meat, thus allowing it to cool more rapidly, it is probable that the infection would be less prevalent. The ideal solution, of course, both in summer and winter months, is to drain the meat of its broth, and refrigerate it immediately after cooking.

Most attention has been focused on the organism as a cause of general outbreaks. The British Ministry of Health (Report, 1964) lists *Cl. welchii* as causing 42 % of all general, but only 3 % of family outbreaks of food-poisoning occurring in England and Wales during 1963. This is probably due to the actual method of contamination and subsequent multiplication; conditions being favourable in institutional environments, in which most general outbreaks occur. Results given earlier in this paper indicate that carriage within a family group is common—72·5 % of positive specimens from aboriginals occurring in 30·8 % of the families investigated. Families with poor hygiene often have two or three members carrying heat-resistant *Cl. welchii* at the one time, while those with good hygiene have little association with the organism. Turner & Wong (1961) found a 63 % carrier rate in persons who, although in hospital, otherwise have very little association with canteen-type foods, and generally live in large family groups under poor hygienic conditions. Such results suggest that *Cl. welchii* must be considered as a source of food-poisoning within the family, particularly families living under poor hygienic conditions. As the infection is mild, and lasts only 24 hr. the average person would not contact his doctor and such a family outbreak would go unnoticed. In hospitals and boarding schools, however, diarrhoea in a large number of persons at the same time can hardly go unnoticed.

SUMMARY

The incidence of heat-resistant *Cl. welchii* in selected classes of a rural population has been investigated. The carrier rate for the general population was low (1·5–6·0 %), but persons associated with communal feeding and poor hygienic conditions were shown to have a much higher carrier rate (15·1–25 %).

Sex and season had little effect on the incidence of the organism, but among aboriginals the carriage was significantly affected by age. This was probably due to eating habits of the different age groups. The carriage appears to be transient; no persons were shown to be permanent carriers of one strain of heat-resistant *Cl. welchii*.

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Prevalence of toxoplasma antibodies in sera from Greece and Africa

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The first serological survey for toxoplasma antibodies in Greece, was carried out on a selected population of different ages by Mercier, Tzamouranis & Crimbithis (1961).Using mainly the dye test they found that 29 of 170 patients had serum titres greater than 1 in 64. Walters (1957) expressed the view that toxoplasmosis, as judged by serological and other evidence, may be widely endemic in W. Africa. Middlemiss (1957), from radiological data, reported that the disease was present in Sierra Leone, Nigeria and N. Rhodesia. Orio, Depoux, Heuls & Ceccaldi (1958) used the complement fixation test to carry out a survey on unselected patients of different age groups in the Middle Congo, and found a relatively high incidence. Positive results at 1/8 were found in 7·4 % of those aged 5–15 years, in 12 % at 15–30 years and in 9 % at above 30 years. De Jongh & de Jager (1959) reported the first case of toxoplasmosis in Liberia. From the results of the dye test Ffrench (1962) concluded that the human disease was prevalent in Ghana. Of 63 sera, 31 had dye test titres of 1/16 or greater. Ludlam (1965) using the same test found a high incidence of toxoplasma antibodies among adult Africans of both sexes in the Niger Delta with a much higher incidence of dye test antibodies (83·3 % at 1/8) in men in the southern part of the Niger Delta than in men from the northern area (52·6 %).

MATERIAL AND METHODS

We have examined sera from healthy members of three different tribes, the Baganda, Masai and Bondei, in East Africa as well as from unselected healthy Negroes from Harbel, Liberia, and in Greeks from the island of Syros and the Greek mainland. Titres were determined by the direct-agglutination (Fulton & Turk, 1959) and dye tests (Sabin & Feldman, 1948; Fleck & Payne, 1963), and are reported below. Large numbers of arthropods obtained from Greece were examined to find if they were harbouring toxoplasma.

Antigen. The RH strain (Sabin, 1941) of the parasite was used in both tests.

Dye test. Sera were stored at − 20° C. and heated at 56° C. for 30 min. just before testing.

Direct agglutination test. A formolized suspension of *Toxoplasma gondii* free from other cells served as antigen.

* Supported by the Medical Research Council.

Arthropods. Six hundred mosquitoes, either culicine or anopheline (*Anopheles maculipennis*, *A. superpictus*, *A. saccharovi* and *A. hyrcanus*), as well as 260 bed-bugs and 400 ticks were obtained from various malaria stations on the Greek mainland. They were ground in 10% rabbit serum in normal (0·85%) saline and the suspensions were examined microscopically for the presence of *T. gondii* and injected intraperitoneally into mice. The peritoneal contents of the mice were examined at 4 days and again at 8 days.

RESULTS

Table 1 shows the titres of 228 sera grouped according to age, from an unselected population of Greeks living on the island of Syros and on the mainland. The sera were obtained from volunteer army recruits, healthy pregnant women, hospital staff and school-children. They are now grouped together since the titres found for both communities were approximately the same. The rising incidence of anti-

Table 1. *Dye tests on sera from a healthy Greek population*

	Age in years					
	0–10	–20	–30	–40	40+	Totals
Sera positive at						
1/1024	2	1	0	1	0	4
1/256	3	12	25	12	2	54
1/64	1	7	15	7	12	42
1/16	1	2	12	14	8	37
1/4	0	6	5	1	7	19
Sera negative	9	26	22	12	3	72
Total						228
Percentage positive at 1/16 or more	44	41	66	72	69	60

Table 2. *Dye tests on sera from a healthy Liberian population*

	Age in years			
	11–20	21–30	30+	Totals
Sera positive at				
1/1024	2	1	0	3
1/256	5	8	2	15
1/64	7	21	9	37
1/16	3	15	11	29
Sera negative	9	30	10	49
Total				133
Percentage positive at 1/16 or more	65	60	69	63

body with increase in age is indicated and is commonly found in surveys of this nature. There is a levelling from the age of 30 years onwards. The incidence of antibody in this population is higher than that found in Britain for all age groups (Beattie, 1958; Fleck, 1963).

The results obtained with 133 Liberian sera are given in Table 2. The population was healthy and again unselected. The incidence of antibodies was high under the

age of 20 years and did not rise with increase in years—an unusual finding. The numbers of sera from 30 years upwards are too few to allow speculation.

The results for seventy-two sera of three East African tribes shown in Table 3 are too few in number to allow any conclusion to be drawn other than that antibodies were present among these populations, with only a moderate increase in incidence with age.

Table 3. *Dye tests on sera from healthy East Africans*

	Bondei infants (½–2 years)	Bondei adults (over 20)	Masai infants	Baganda adults
Sera positive at				
1/256	0	0	0	3
1/64	1	3	1	7
1/16	1	0	2	10
1/4	1	0	0	3
Sera negative	10	4	6	20

Table 4. *Comparison of dye test and direct agglutination titres in sera from Greece and Liberia*

Direct agglutination titre	Neg. to 1/8	1/16–1/32	1/64–1/128	1/256–1/512	1/1024 or more
1/5120 or more	0	0	0	0	2
1/1280–1/2560	0	1	8	16	2
1/320–1/640	4	13	68	6	1
1/80–1/160	46	106	40	1	0
Neg. to 1/40	149	61	7	1	0

Dye-test titre

In Table 4 the results obtained by the direct agglutination and dye tests for a total of 532 Greek and Liberian sera are compared. The extra 171 sera in this analysis were obtained from Greek hospital patients. There is reasonably good correlation between the tests, the direct test giving titres approximately 4–5 times that obtained in the dye test, corresponding to a difference of 1 dilution used in the latter test. All the sera found negative in the dye test gave direct agglutination titres of 1/10 to 1/40. The agglutination test has a number of advantages over the dye test in that it is macroscopic in character, avoids the need for live parasites and accessory factor, and can be rapidly performed with an antigen which remains stable over 6 months.

In the examination of arthropods from Greece for the presence of toxoplasma a total of 639 mice were used for direct inoculation with ground-up arthropod material or for subinoculation with a brain suspension when the presence of infection in the original mice was suspected. Direct agglutination tests performed on the sera of forty-nine inoculated mice and on controls indicated that infection was absent. Difficulty was experienced when the suspension became infected with bacteria or fungi. This was prevented by storing the suspension in 1:200 formol saline.

DISCUSSION

It is clear from the results recorded that antibodies to toxoplasma are present among all the populations examined. It was not possible to select the samples of sera so that the infection rates in different classes of these populations could be determined as a help in providing clues to the possible mode of spread of the disease. In the past, serological surveys of this nature have shown that antibodies have a widely varying incidence throughout the world. In one area in Guatemala it was 96 %, in Tahiti 68 % and 30 % in Europe and N. America, but only 4 % among Navajo Indians in Arizona. The reason for the considerable variation in incidence is not understood, especially as the mode of spread of the disease has so far eluded investigators. Differences in climatic conditions such as rainfall and temperature may exert an influence on the proportion of the population affected, so also may closeness of association with wild or domestic animals. In both these groups the disease is highly endemic and is regarded as a zoonosis. Arthropods, however, have not yet been incriminated as vectors in nature, and our results support earlier findings. Recent work by Hutchison (1965) has indicated a possible method of spread of the disease which may be applicable to man. It is known, for example, that infection can be transmitted in animals by eating infected food. This author has suggested that *T. gondii* free in the intestine from food or from lesions may associate with nematode ova and thus pass to the exterior. Critical experiments are now being carried out to find if the infective material was indeed transmitted by helminth ova.

SUMMARY

Sera from healthy Greeks, Liberians and East Africans were tested for dye test and direct agglutinating toxoplasma antibodies. At a titre of 1/16 or more 137/228 Greeks, 84/133 Liberians, 2/13 Bondei infants, 3/7 Bondei adults, 3/9 Masai infants and 20/43 Baganda adults gave a dye test positive result. This confirms the widespread occurrence of this infection.

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Studies on the carrier state of cattle exposed to foot-and-mouth disease virus

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The experimental and epizootiological evidence for the existence of a carrier state in cattle following exposure to foot-and-mouth disease virus (FMDV) has been reviewed by van Bekkum, Frenkel, Frederiks & Frenkel (1959). These workers also reported their own work which showed that virus could be detected regularly in the 'saliva' from a rather large proportion of cattle over a period of several months after recovery from clinical disease. These findings have been confirmed by Sutmöller & Gaggero (1965) during the course of studies undertaken to develop suitable techniques for recovery of virus from field outbreaks of disease when vesicular material was no longer available. Both groups of workers used a sampling instrument to collect a fluid specimen from the upper part of the oesophagus, pharynx and mouth. Van Bekkum *et al.* have referred to such specimens as 'saliva'; Sutmöller & Gaggero have preferred the term 'oesophageal fluid'.

The studies reported here were concerned with the measurement of the infectivity of samples taken by this method and the determination of the sites of virus persistence and multiplication in the carrier animal.

MATERIALS AND METHODS

Virus strains

(1) A-119: Pirbright stock cattle strain used at the 25th and 26th cattle passage.
(2) A-Turkey 19/64
(3) SAT 1, SA. 13/61
(4) SAT 3, Bec. 3/64
} World Reference Laboratory samples received from the field and used at the 1st to the 3rd cattle passage.

Cattle

The supply and maintenance of cattle under experiment have been described by Henderson (1952). Cattle retained for periods longer than 6 weeks after exposure to virulent virus were washed thoroughly and moved to a clean Isolation Unit.

The previous experimental history of these cattle was such that they could be placed in one of two categories:

A: Animals which had been vaccinated with inactivated FMD virus vaccines and then challenged 20–21 days later by tongue inoculation of $10^{5\cdot0}$ ID_{50} of virulent virus. The majority of these animals had possessed sufficient immunity to modify the development of local lesions considerably and to prevent the development of secondary lesions completely.

B: Animals which had developed generalized FMD. These had been used for the passage or titration of the above virus strains or challenged as susceptible controls in vaccine evaluation trials.

Collection and handling of samples

Food was withheld from the animals for 18 hr. before taking samples.

Saliva

Saliva was collected by means of a disposable syringe from the region between the lateral surface of the tongue and the mandibular cheek teeth.

Oesophageal/pharyngeal sample

The collecting beaker or cup referred to by van Bekkum *et al.* (1959) and recently described by Sutmöller & Gaggero (1965) was passed by mouth into the oesophagus for a distance of about 6 in. As it was being withdrawn, attempts were made to contact both lateral walls of the pharynx. The volume of the sample collected varied from 5 to 10 ml. and consisted mainly of saliva. Varying amounts of flocculating mucus and cell debris were present and on occasion small hair-balls and traces of regurgitated rumen contents. Immediately after collection each sample was added to an equal volume of diluent and the mixture shaken vigorously.

Post-mortem specimens

Superficial scrapings were made of the surface epithelium from 2–12 sites from each animal. The areas sampled are described in the terminology used by Sisson & Grossman (1945) and included the dorsal and ventral turbinates and the posterior part of the nasal septum; the anterior part of the dorsum, the dorsal prominence and the lateral surface of the tongue; the glosso-epiglottic space and the root of the tongue; the lateral and dorsal surfaces of the pharynx; the dorsal and ventral surfaces of the soft plate; the glandular, cervical and thoracic regions of the oesophagus; the cervical and thoracic regions of the trachea; and the wall of the urinary bladder. In addition, the contents of the tonsillar sinuses and the bulk of the lateral and central zones of each tonsil were collected from the majority of animals.

No attempt was made to clean or wash the tissues of the first six animals examined *post mortem* but thereafter the tissues were washed thoroughly in running water before sampling began and after taking each specimen.

Each specimen was added to a standard volume of diluent which was later increased if necessary to give a final dilution of 1/10 (v/v) of the original harvest as estimated by a packed cell volume. The scrapings of epithelium and the contents of the tonsillar sinuses were processed by grinding in a Griffith's tube and the resultant suspension assayed for infectivity. The tonsils were ground with sand and only the supernatant fluid screened for infectivity.

All samples were held at room temperature and assayed for infectivity within 2–3 hr. of collection or of the slaughter of the animal.

Diluent

Phosphate buffered saline (PBS) was used as a diluent for all purposes. Antibiotics were added to give final concentrations of penicillin 1000 units/ml., streptomycin 400 μg./ml., polymyxin B 100 units/ml., neomycin 140 μg./ml. and mycostatin 100 units/ml.

Isolation and infectivity assay of virus

Plaque assay

Samples were examined for plaque-forming units in baby hamster kidney (BHK) cell strain monolayers (Mowat & Chapman, 1962); 0·2 ml. volumes of the sample were placed on 5–8 BHK 2-day-old monolayers in 60 mm. petri dishes and after an adsorption period of 1 hr. at 37° C. a nutrient agar overlay was added. The monolayers were incubated for 48 hr., stained with a 1/10,000 dilution of neutral red in PBS and plaques were counted. When recovery of the virus was required for confirmation of type or other tests a 1/20,000 dilution of neutral red in a nutrient agar overlay was used, plaques were picked and the virus grown up in BHK bottle cultures.

Mouse inoculation

Groups of 20–30 6-day-old randomized Pirbright-strain mice were inoculated intraperitoneally with 0·1 ml. volumes of the sample. Mice were observed for 8 days and mice dying after 24 hr. were checked for complement-fixing virus antigen either directly or after a further passage.

Serum neutralization tests

The cell metabolic inhibition test (colour test) using primary monolayers of pig kidney cells (Martin & Chapman, 1961) was used. The neutralization titres are expressed as the log. reciprocal of the final dilution of serum present in the serum virus mixture at the 50% end-point (estimated according to the method of Kärber, 1931). Homologous tissue culture adapted virus strains were used in these tests.

RESULTS

The infectivity of oesophageal/pharyngeal samples

Details of the frequency of recovery of virus and the titres measured in samples collected from a group of ten cattle after infection with the A-119 virus are shown in Table 1.

The figures presented are those obtained from plaque counts in BHK monolayers. The presence of virus in the majority of these samples was confirmed by recovery of virus in mice. Occasionally, samples with a high infectivity in BHK cells were negative in mice and, conversely, samples negative in BHK cells proved positive in mice. In general, samples with a plaque count of up to 5/ml. killed up to 15% of mice inoculated, samples with counts of 15–50/ml. killed on average 40%, and samples with plaque counts greater than 100/ml. killed 80–100% of mice. Any comparison of the sensitivity of the two methods used for isolating virus

must be related to the volumes of the sample tested. As a routine, 2·0 ml. of each sample were inoculated into mice and 1·0 ml. into BHK monolayers. Another factor which would affect sensitivity is the ability of the assay system to detect partially neutralized virus. Hyslop (1965) has shown that neutralization indices (measured in mice) of saliva collected from challenged animals can range from 1·19 to 3·7. No direct experiment was made to estimate the neutralizing activity

Table 1. *The frequency of recovery of virus and the infectivity of oesophageal/pharyngeal samples from ten cattle convalescent after clinical infection with FMD virus A-119*

Weeks after infection	No. of cattle yielding positive samples	Infectivity of samples	
		Mean	Range
2	9	2·1*	1·3–3·0
3	10	2·4	1·7–3·0
4	10	2·1	1·3–2·9
5	10	2·0	1·2–2·9
6	9	1·8	1·2–2·9
9	9	1·3	0·6–2·0
10	7	1·4	0·3–1·8
11	9	1·3	0·3–2·4
12	8	0·9	0·3–2·0
13	7	1·5	0·8–1·9
14	7	1·2	0·3–2·1
17	7	1·4	0·9–1·8
19	6	1·4	0·9–1·8
22	5	0·8	0·3–1·9
26	5	1·0	0·3–2·0

* Log_{10} pfu/ml. of undiluted sample.

Table 2. *Recovery of virus from oesophageal/pharyngeal samples collected from cattle convalescent after clinical infection with FMD virus strains*

Virus	Period of sampling: weeks after infection	No. of occasions sampled*	No. of cattle in group	No. of samples from which virus recovered	Comments
A-Turkey	3 to 21	12	4	40	1 animal yielded virus on all occasions, 3 animals intermittently
SAT 1	2 and 3	2	4	8	All animals yielded virus on both occasions
SAT 3	14 to 25	9	6	9	Only 3 animals yielded virus during this period
SAT 3†	2 to 8	7	6	33	3 animals yielded virus on all occasions, 3 animals intermittently

* Intervals between sampling varied from 1 to 3 weeks.
† Virus recovered from carrier cattle 14 and 18 weeks after infection.

of oesophageal/pharyngeal samples in mice or BHK monlayers. However, the results of repeat titrations in BHK cells of samples after storage at 4° C. showed a five-fold reduction in titre to have occurred after 2 days storage (mean of ten samples) and a 20-fold reduction in titre after 7 days (mean of 7 samples). This loss in infectivity may have resulted from the presence of low levels of neutralizing antibody in the samples or from inactivation due to the high pH of these samples (mean pH $8{\cdot}24 \pm 0{\cdot}16$).

Similar statistics to those shown in Table 1 were collected for four other groups of cattle after infection with three different strains of virus. This work is summarized in Table 2. The results given in Tables 1 and 2 indicate that these strains of FMDV can persist and multiply in the majority of cattle for an extended period following clinical infection. The infectivity of samples collected from the SAT 3 and A-Turkey cattle remained fairly low and only on two occasions were samples collected which contained 100 pfu/ml. In contrast, some of the animals convalescent to the A-119 strain yielded samples between the 2nd and 6th week with infectivities up to 1000 pfu/ml.

Van Bekkum *et al.* (1959) reported that vaccinated cattle exposed to virulent virus could also become carriers. This was confirmed in a group of cattle which had been challenged 20 days after vaccination with an inactivated A-119 vaccine. Twenty of 21 animals sampled 2 weeks after challenge yielded positive samples (mean infectivity—$\log_{10}$ pfu/ml. 2·1, range 0·9–3·0) 8 of 8 at 3 weeks (2·2, range 1·3–2·8) and 5 of 5 at 4 weeks (2·0, range 1·6–2·6). The geometric mean pre-challenge antibody level of these cattle was 1·69 and their immune status sufficient to prevent the development of secondary lesions following challenge. These figures found for the immune animals are similar to those for fully susceptible cattle (Table 1). Thus, the possession of immunity at the time of challenge would not appear to reduce the incidence or lessen the intensity of the carrier state.

Sites of multiplication of virus in the carrier animal

The recovery of virus in relatively high titre from carrier animals over several months must indicate continual multiplication of virus at some site or sites in the animal. The high serum antibody levels of these animals suggest that the source of infectivity is in direct or indirect communication with the upper digestive and respiratory tracts. Some workers, e.g. Cottral, Gailiunas & Campion (1963), have interpreted the results of van Bekkum *et al.* (1959) as suggesting that the source of the virus was the salivary glands. Saliva samples taken from the mouth of eighteen animals convalescent after infections with three of the virus strains used in these studies were all negative for virus when assayed in BHK cells, but oesophageal/pharyngeal samples taken immediately afterwards were all positive. Hyslop (1965) also failed to detect infectivity in saliva samples taken from the mouths of twelve steers 5 weeks after infection. On the basis of these findings, no examination of the salivary glands for virus was undertaken in the present series of experiments. Cottral *et al.* (1963) studied the persistence of virus in lymph nodes of the heads and carcases of cattle following infection with FMDV. They obtained some evidence that virus persisted for up to 15 days but not for 17 days after infection.

P. Sutmöller (personal communication), failed to detect virus in the salivary glands and the lymph nodes of a carrier bullock slaughtered 1 month after challenge. These reports did not indicate that the lymph nodes associated with the upper digestive and respiratory tracts were responsible for the virus detected in oesophageal/pharyngeal samples from carrier animals and lymph nodes were not therefore examined.

The results of infectivity titration of post-mortem specimens from fourteen cattle killed 3–5 weeks after challenge with the A-119 virus are detailed in Table 3. These animals, with the exception of FE 48 and FE 49, had been vaccinated with an inactivated A-119 virus preparation 20 days before challenge. The infectivity titres of oesophageal/pharyngeal samples taken from three of these animals immediately before slaughter have been included in Table 3, as also have the neutralizing antibody titres of the pre-challenge and post mortem serum samples. Virus was recovered from thirteen of the fourteen animals.

The results obtained from forty more cattle killed between 2 and 18 weeks after challenge or infection are summarized in Table 4. Virus was recovered from 18 of 22 cattle killed 2 to 3 weeks after challenge with SAT 1 virus, 4 of 6 animals killed 3 weeks after challenge with A-Turkey virus, 5 of 6 cattle killed approximately 10 weeks and 1 of 6 killed 28 weeks after infection with SAT 3 virus.

The percentage frequency of virus recovery and the mean infectivity levels measured for the positive specimens collected from the forty-one cattle from which virus was recovered *post mortem* are given in Table 5.

These figures were compiled from infectivity assays in BHK cells. The results of mouse inoculation did not support these figures. Samples from the pharynx and dorsal surface of the soft palate which gave infectivity levels of up to 1000 pfu/ml. killed only a small percentage of mice inoculated. This can be explained by the ability of the mouse to detect minimal levels of antibody. It was found that supernatant fluids of suspensions prepared from pharyngeal scrapings from carrier animals possessed considerable neutralizing activity. When the homologous virus was added to such fluids and incubated at 37° C. for 1 hr., infectivity was reduced 30-fold when measured in BHK and up to 5000-fold when measured in mice. Similar tests with these tissue supernatants using a virus of different immunological type resulted in no reduction in infectivity for BHK and only a fourfold drop in infectivity when titrated in mice.

The results indicate that the mucosae of the pharynx and the dorsal surface of the soft palate (which forms the anterior floor of the pharynx) are the main sites of virus multiplication in the carrier animal. Other regions contiguous or in close proximity to these areas also yielded virus on occasion but at much lower titres. Whether these recoveries represent additional sites of virus multiplication is debatable. Attempts to relate the amount of virus recovered from any particular site with the importance of that site to the carrier state must take account of the nature of the specimen assayed. Specimens from the pharynx and dorsal surface of the soft palate contained the bulk of the surface epithelium and also some sub-epithelial lymphoid tissue. Such specimens possessed considerable neutralizing activity and the infectivity levels recorded for these sites were almost certainly

Table 3. *Infectivity titration of specimens obtained from cattle* 21–37 *days after challenge with A*-119 *virus*

Days after challenge...	21		23		28		29	31		35			37	
Cattle no. (prefix FE)	23*	26*	2*	20*	6*	19*	24	28	98	30	48	49	90	99
Oesophageal/pharyngeal sample (*ante mortem*)†	NT	NT	NT	NT	1·8	1·8	2·3	NT	NT	NT	NT	NT	NT	NT
Tongue	0·0	1·0	0·0	1·4	NT	NT	NT	NT	NT	0·0	0·0	0·0	0·0	0·0
Glosso-epiglottic space	0·0	1·7	0·0	0·0	0·0	1·6	0·8	0·0	1·1	0·0	0·0	0·0	NT	NT
Pharynx	0·0	1·9	2·3	1·1	2·6	3·0	2·7	2·0	2·9	0·0	2·2	2·1	0·0	2·0
Dorsal surface soft palate	NT	NT	1·8	2·1	2·7	3·2	2·5	2·8	2·7	1·0	2·2	1·7	2·7	2·2
Ventral surface soft palate	NT	NT	1·9	0·0	1·6	1·6	0·8	0·0	0·0	0·0	0·0	0·0	NT	NT
Tonsillar sinuses	0·0	1·3	1·1	0·0	1·4	0·0	1·2	1·9	0·0	0·0	NT	0·0	NT	NT
Tonsils	NT	1·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	NT	NT
Glandular region—oesophagus	0·0	0·0	0·0	0·0	2·5	2·1	0·0	0·0	1·1	0·0	0·0	0·0	0·0	0·0
Remainder of oesophagus	0·0	0·0	0·0	0·0	1·1	0·0	0·8	0·0	0·0	NT	NT	NT	NT	NT
Turbinates	NT	NT	NT	NT	0·0	0·0	0·0	NT	NT	NT	NT	NT	NT	NT
Trachea	0·0	0·0	2·0	0·0	0·0	0·0	0·0	0·0	0·0	NT	NT	NT	NT	NT
Urinary bladder	NT	NT	NT	NT	NT	NT	NT	NT	NT	0·0	0·0	0·0	NT	NT
Pre-challenge serum titre‡	1·8	1·5	1·7	1·9	1·5	2·0	1·5	1·8	2·0	2·3	0·6	0·5	1·7	1·5
Post-mortem serum titre‡	3·0	3·7	3·7	4·2	NT	NT	3·7	3·0	3·1	3·6	3·0	3·0	3·1	3·3

* Tissues not washed before collection of specimens.
† Log_{10} pfu/g. or ml. of undiluted specimens.
‡ Log reciprocal serum dilution.
NT, Not tested.

considerably underestimated. Specimens taken from the tongue, glosso-epiglottic space and oesophagus contained less cellular material and possessed relatively little neutralizing activity. The irregular occurrence and low levels of virus recovered from these areas could represent contamination from the pharyngeal region.

Table 4. *Frequency of virus recovery and mean infectivity of specimens taken* post mortem

Virus...	SAT 1		A-Turkey		SAT 3			
Number of cattle	22		6		6		6	
Days after infection	14–21		21		62–75		196	
Tongue	3/22*	1·7†	NT	—	0/2	—	NT	—
Glosso-epiglottic space	8/22	1·9	1/3	1·0	0/4	—	NT	—
Pharynx	11/22	1·5	4/6	2·2	4/6	1·8	0/6	—
Dorsal surface soft palate	18/22	2·4	4/6	2·3	4/6	2·0	1/6	1·6
Ventral surface soft palate	8/22	1·6	NT	—	1/2	1·0	NT	—
Tonsillar sinuses (2)	4/44	1·6	2/12	1·1	0/12	—	0/12	—
Tonsils (2)	NT	—	0/12	—	0/12	—	0/12	—
Glandular region oesophagus	2/22	1·4	1/6	1·0	0/2	—	NT	—
Remainder of oesophagus	NT	—	NT	—	0/2	—	NT	—
Turbinates	NT	—	NT	—	0/2	—	NT	—
Trachea	4/22	1·3	1/3	1·0	0/2	—	NT	—
Urinary bladder	NT	—	NT	—	0/2	—	NT	—

* No. of positive specimens/no. of specimens tested.
† Mean infectivity of positive specimens ($\log_{10}$ pfu/g. or ml. of undiluted specimen).
NT, Not tested.

Table 5. *Percentage frequency of virus recovery and mean infectivity of specimens collected from forty-one cattle from which virus was recovered* post mortem

Specimen	Number examined	Percentage frequency virus recovery	Mean infectivity of positives
Dorsal surface of soft palate	40	97	2·26*
Pharynx	41	73	1·87
Ventral surface of soft palate	30	43	1·49
Glosso-epiglottic space	36	36	1·66
Tonsillar sinuses	38	29	1·43
Tongue	25	20	1·52
Trachea	30	20	1·37
Glandular region of oesophagus	35	17	1·60
Remainder of oesophagus	10	20	0·95
Tonsils	21	5	1·00

* $\log_{10}$ pfu/g. or ml. of undiluted specimen.

DISCUSSION

The results presented, in conjunction with the findings of van Bekkum *et al.* (1959) and Sutmöller & Gaggero (1965), suggest that the carrier state in cattle may be a normal sequel to infection with virulent strains of FMDV. The four virus strains

used in these investigations were examples of differing epizootiological origin. The SAT 3 strain was recovered from a very mild outbreak of disease in Bechuanaland and only produced minor clinical lesions in a small proportion of animals at risk (R. S. Hedger, personal communication). The virus, however, proved to be fully virulent for cattle in tests at this Institute. The SAT 1 strain was recovered from the 1961 enzootic in South Africa, some details of which have been recorded by Galloway (1962). The A-Turkey virus was recovered from the 1963–65 epizootic in the Middle East which resulted in a severe, widespread infection of cattle. The A-119 strain can be classed as a virulent laboratory strain in that it has been maintained by passage in cattle since its recovery from a British field outbreak in 1932.

The superficial nature of the virus infection of the pharyngeal region of carrier animals, together with the relatively high infectivity found in some samples taken from the living animal, must constitute a potential hazard to susceptible animals maintained in close contact. The physical action of regurgitation could be expected to bring some infective material forward into the oral cavity and so allow contamination of the environment. However, van Bekkum *et al.* (1959) have given ample evidence of the non-infectivity of these animals for in-contact susceptibles. In a small experiment carried out at this Institute, four susceptible cattle were housed in close contact with six SAT 3 animals (Table 2, group 3) from the 9th to the 14th week of convalescence. At the end of this period no clinical or serological evidence of transmission of infection was obtained and no virus could be recovered from oesophageal/pharyngeal samples taken on three occasions from the four susceptibles.

Multiplication of virus in an immune animal over a period of several months might be expected to result in some change in the characteristics of the virus both in respect of virulence and of antigenic properties. Preliminary studies with two strains of the SAT 3 virus recovered after 14 weeks in carrier animals have shown that no major changes have occurred. This aspect of the work is continuing to see whether such changes can result from a prolonged carrier state.

SUMMARY

Cattle infected with FMDV strains of different epizootiological origin developed a carrier state which persisted in the majority of animals for several months. Fluid samples taken from the oesophageal/pharyngeal region were assayed for infectivity by plaque counts on BHK monolayer cultures and by mouse inoculation. With one strain of virus, infectivity levels of up to 1000 pfu/ml. were recorded for several weeks after infection but in general the virus content of samples was below 50 pfu/ml.

The sites of virus persistence and multiplication were identified by titration of suspensions of mucosae and epithelia taken *post mortem*. Virus was recovered from 41 of 54 cattle killed 14–196 days after infection. The chief sites of virus multiplication based on the frequency of virus recovery and infectivity titres were the dorsal surface of the soft palate and the pharynx. Virus was recovered less

frequently from the ventral surface of the soft palate and the glosso-epiglottic space and only occasionally from the tonsillar sinuses, tonsils, tongue, trachea and oesophagus. No virus was detected in the turbinates or in the epithelium of the urinary bladder.

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A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production

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A property of mycoplasmas (PPLO) is the inhibition of their growth by specific antibody. Edward & Fitzgerald (1954) first demonstrated growth inhibition with antiserum incorporated into the agar medium and Nicol & Edward (1953) used the technique to study mycoplasma strains isolated from the urogenital tract. In order to type mycoplasma strains this technique was modified by several workers. Huijsmans-Evers & Ruys (1956) placed filter-paper disks soaked in specific antisera on the agar surface. Herderscheê, Ruys & van Rhijn (1963) used drops of antisera on the agar and Clyde (1964) evaluated and standardized the filter-paper-disk method. However, these techniques are relatively insensitive for the quantitative measurement of antibody, especially in human sera. A technique which involved sampling and titration of antiserum–organism suspensions was used successfully by Bailey *et al.* (1961) but it is laborious. A similar procedure was used by Jensen (1963) for measuring growth-inhibiting antibody to *Mycoplasma pneumoniae*. A simpler quantitative colour method for *M. pneumoniae* has also been described by Jensen (1964). This is based upon the inhibition of reduction of tetrazolium. Unfortunately, this technique does not have wide application since *M. pneumoniae* is unique among the mycoplasma species that infect man in its ability to reduce readily tetrazolium salts. However, the growth of *M. pneumoniae* and certain other mycoplasma species is accompanied by the production of acid, and the incorporation of phenol red into the medium permits the resultant pH change to be seen. The addition of specific antiserum to the medium inhibits mycoplasma growth and this inhibition can be visualized by failure of the indicator to change colour. This communication describes the development of this technique for the quantitative measurement of growth-inhibiting antibody to the acid-producing mycoplasmas and indicates its practical significance.

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MATERIALS AND METHODS

Mycoplasmas

The following mycoplasma strains were chosen for this study. The FH strain of *M. pneumoniae* was obtained from Liu (1957) and was subcultured over 200 times in this laboratory on agar or in broth medium. The 1428 strain of *M. pneumoniae* was recovered from a marine recruit with atypical pneumonia (Couch, Cate & Chanock, 1964) and was subcultured three times on agar medium. *M. fermentans* (strain G) was obtained from D. G. ff. Edward. The Negroni agent was isolated during the course of attempts to isolate viruses from human leukaemic bone marrow and was originally considered to be a virus (Negroni, 1964). Further studies demonstrated that it was a mycoplasma (Girardi, Hayflick, Lewis & Somerson, 1965). The source of other prototype mycoplasma strains used for the production of antisera has been described previously (Taylor-Robinson, Somerson, Turner & Chanock, 1963).

Media

The medium used for the maintenance of organisms has been described previously (Taylor-Robinson *et al.* 1963). It consisted of seven parts of Difco PPLO agar or broth, two parts of unheated horse serum, one part of 25 % yeast extract, 1/2000 thallium acetate, and 1000 units of penicillin G/ml. Henceforth this medium will be termed 'standard medium'. The medium used for the growth inhibition tests and for the growth of organisms to be used in the inhibition tests was the standard medium with 1 % glucose and 0·002 % phenol red (final concentrations) and the final pH was adjusted to 7·8 with hydrochloric acid. Henceforth this medium will be termed 'standard medium with additives'. Organisms for rabbit immunization were grown in a medium of rabbit infusion broth supplemented with 5 % rabbit serum. The preparation of this infusion has been described previously (Taylor-Robinson *et al.* 1963).

Growth of organisms for use in inhibition tests

One hundred ml. amounts of standard broth medium with additives were inoculated with suspensions of mycoplasmas in their early phase of growth and incubated at 34° C. until the pH of the medium decreased by about half a pH unit (i.e. pH 7·8 to about 7·3). The broth cultures were then divided into 1·0 ml. amounts and stored at −70° C. The materials were titrated by making serial tenfold dilutions in the standard medium with additives. These dilutions were incubated at 34° C. in screw-capped vials or microtitre plates. The titre was considered to be the highest dilution which produced a colour change when the test was read at a time when colour changes were no longer progressive.

Rabbit antisera and human sera

Rabbit antisera were prepared as described in detail previously (Taylor-Robinson *et al.* 1963). Briefly, all organisms except *M. pneumoniae* were grown in rabbit infusion broth supplemented with rabbit serum and 20-fold concentrates

were used to inoculate rabbits; *M. pneumoniae* was grown in chick embryo lung and the homogenized suspension of lung used to inoculate rabbits. Paired human sera were obtained from adult male volunteers, before and 3 weeks after they had been inoculated via the oropharynx with the FH strain of *M. pneumoniae*. Sera were obtained also from adult patients, mostly 30–59 years of age and a few older, with non-respiratory illnesses at D.C. General Hospital, Washington, D.C.

Complement-fixation (CF), indirect-haemagglutination (IHA) and tetrazolium reduction inhibition (TRI) techniques

The method used for CF has been reported before (Taylor-Robinson *et al.* 1963). Eight units of antigen were used and the tests were performed by overnight fixation at 4° C. in micro CF plates. The method used for IHA was that described previously (Taylor-Robinson, Canchola, Fox & Chanock, 1964) but modified by the use of sensitized sheep erythrocytes stored at −70° C. (Taylor-Robinson, Ludwig, Purcell, Mufson & Chanock, 1965). The TRI test for measurement of antibody to *M. pneumoniae* was essentially that described by Jensen (1964). Briefly 0·025 ml. amounts of serum diluted in standard medium supplemented with 0·05% triphenyl-tetrazolium chloride were mixed with 0·025 ml. of various dilutions of mycoplasma organisms in the same medium. To this was added a final 0·15 ml. of the same medium. With growth of the organism, colourless tetrazolium is reduced to a red formozan; specific antibody inhibits growth and the resultant colour change.

Growth inhibition or fermentation inhibition (FI) test for acid-producing mycoplasmas

Tests were performed in disposable plastic microtitre plates with U-shaped cups manufactured by Cooke Engineering Company, Alexandria, Virginia. These plates were not treated with ultraviolet radiation before use and no special precautions were taken to prevent bacterial contamination during the performance of the test. Sera were diluted 1/2·5 or 1/5 in standard medium with additives and used after heating at 56° C. for 30 min. In some instances the serum specimens were tested without prior heating. After the addition of 0·025 ml. (1 drop) of standard medium with additives to each cup, serial twofold dilutions of serum were made with spiral wire loops. Mycoplasma suspensions diluted as required in the same medium were then added in 0·05 ml. amounts to each cup and the total volume made up to 0·2 ml. by the addition of 0·125 ml. (5 drops) of standard medium with additives. When guinea-pig serum was used, it was added in the final 0·125 ml. of medium. Controls consisted of cups which contained a mixture of 0·05 ml. of mycoplasma organisms and 0·15 ml. of standard medium with additives. Plates were sealed with 'Scotch Brand' cellophane tape and were not agitated. Incubation was at 34° C. under aerobic conditions unless stated otherwise. Colour changes were observed by placing the plates over a mirror with a fluorescent white light above the plates. An arbitrary system of classifying the colour changes was as follows: no change −(pH 7·8); + (pH *ca.* 7·3); + + (pH *ca.* 6·8); + + + (pH *ca.* 6·2 or less). The highest serum dilution which prevented a colour change of greater than

50 % when compared with the same dilution of organisms grown in the absence of antiserum was considered the end-point of the serum antibody titration. In some instances, slight colour changes were observed in cups which contained only standard medium. This colour change could be abolished without interfering with changes produced by growth of the mycoplasmas by removing the cellophane tape from the plate and reincubating at 34° C. for a few hours.

Definition of terms used

One colony-forming unit (cfu) is contained in the highest dilution of a mycoplasma suspension that will produce one colony on a PPLO-agar plate.

One colour-changing unit (ccu) is contained in the highest dilution of a mycoplasma suspension that will produce a change of half a pH unit in standard broth medium with additives at a specified time.

Table 1. *Microtitre plate colour change titration of* Mycoplasma pneumoniae

M. pneumoniae suspension		Approx. pH of medium in cup on indicated day of incubation						
Dilution	No. of ccu* at indicated dilution	3	4	5	6	7	8	10
10^{-2}	10^4	7·3	6·8	6·2	6·2	6·2	6·2	6·2
10^{-3}	10^3	7·8	7·8	7·0	6·2	6·2	6·2	6·2
10^{-4}	10^2	7·8	7·8	7·8	7·3	6·5	6·2	6·2
10^{-5}	10	7·8	7·8	7·8	7·8	7·5	6·5	6·2
10^{-6}	1	7·8	7·8	7·8	7·8	7·8	7·8	6·5
10^{-7}	0	7·8	7·8	7·8	7·8	7·8	7·8	7·8
Highest dilution at which pH change of half a unit (1 ccu) occurred on indicated day		10^{-2}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-6}

* Colour changing units/0·05 ml. of original suspension calculated at day 10.

RESULTS

Microtitre plate titration of mycoplasmas

Tenfold dilutions of *M. pneumoniae* in standard medium with additives were dropped in 0·05 ml. amounts into cups of a microtitre plate and 0·15 ml. of the same medium was added to each cup. The plate was incubated at 34° C. aerobically and the results recorded at various times of incubation are shown in Table 1. On the third day of incubation one ccu was contained in a 10^{-2} dilution, i.e. a 10^{-2} dilution of the *M. pneumoniae* suspension contained in 0·05 ml. produced a colour change of half a pH unit (7·8–7·3). On the tenth day one ccu was contained in the 10^{-6} dilution, i.e. a 10^{-6} dilution of the *M. pneumoniae* suspension was the highest dilution which produced a colour change. Titration of the same *M. pneumoniae* suspension on PPLO-agar plates showed that it contained 3×10^6 cfu/0·1 ml. so that the titre of the suspension in cfu and ccu at 10 days was approximately the same.

Tests with homologous rabbit antisera

M. pneumoniae (FH strain), *M. fermentans* and the Negroni agent were tested with their respective pre-inoculation and post-inoculation rabbit sera which were used without heat inactivation. 'Block' titrations were performed by diluting the organisms in serial tenfold steps and the sera in serial twofold steps. Table 2 shows the maximum fermentation inhibition (FI) serum titres obtained during

Table 2. *Titre of pre-inoculation and post-inoculation rabbit sera in FI tests with homologous mycoplasma organisms**

Mycoplasma used in test	Titre obtained with: Unheated pre-inoculation rabbit serum	Unheated post-inoculation rabbit serum
M. pneumoniae	10	5120 or >
M. fermentans	< 10	5120
Negroni agent	< 10	< 320

* Tests performed with standard medium containing 20 % unheated horse serum.

Table 3. *Association between colour change and mycoplasma growth*

Mycoplasma tested	Rabbit serum and dilution (reciprocal) tested	Fluid in cup of microtitre plate: Approx. pH	Mycoplasma cfu/ml.
M. pneumoniae	No serum	6·8*	$3{\cdot}3 \times 10^8$
	Pre-inoculation 10	6·5	$1{\cdot}6 \times 10^8$
	Post-inoculation 10240	7·0	$1{\cdot}8 \times 10^8$
	Post-inoculation 5120	7·3	$6{\cdot}0 \times 10^7$
	Post-inoculation 2560†	7·5	$1{\cdot}4 \times 10^7$
	Post-inoculation 640	7·8	$1{\cdot}4 \times 10^4$
M. fermentans	No serum	7·3‡	$6{\cdot}0 \times 10^6$
	Pre-inoculation 10	7·3	$1{\cdot}0 \times 10^6$
	Post-inoculation 2560	7·5	$1{\cdot}7 \times 10^6$
	Post-inoculation 1280†	7·7	$8{\cdot}0 \times 10^4$
	Post-inoculation 320	7·8	$< 1{\cdot}0 \times 10^2$
Negroni agent	No serum	7·3‡	$9{\cdot}0 \times 10^7$
	Pre-inoculation 10	7·3	$5{\cdot}0 \times 10^7$
	Post-inoculation 20480	7·5	$3{\cdot}0 \times 10^7$
	Post-inoculation 10240†	7·7	$3{\cdot}0 \times 10^6$
	Post-inoculation 2560	7·8	$2{\cdot}0 \times 10^4$

* Fluids in the cups of this test titrated after 4 days incubation at 34° C.
† This dilution represented the serum end-point at the time the test was read.
‡ Fluids in the cups of this test titrated after 3 days incubation at 34° C.

the course of readings taken over several days. Unheated rabbit antisera to *M. pneumoniae* and *M. fermentans* inhibited to high titre the growth of their homologous organisms as indicated by the inhibition of colour change. On the other hand, rabbit antiserum to the Negroni agent at the lowest dilution used did not inhibit

the colour change produced by growth of the organism. As will be shown later, it was necessary to add unheated guinea-pig serum to demonstrate the inhibitory activity of Negroni antiserum. All these tests were performed under aerobic conditions. In an atmosphere of 100 % nitrogen, the growth of *M. pneumoniae* was suppressed, but that of *M. fermentans* was not affected. Under anaerobic conditions the titre of the *M. fermentans* antiserum did not increase. In all further experiments aerobic conditions were employed.

Association of colour change with mycoplasma growth

Microtitre plates which contained *M. pneumoniae*, *M. fermentans* and the Negroni agent were incubated at 34° C. At various time-intervals the cups were sampled, the fluids diluted in tenfold steps and inoculated on to PPLO-agar plates so that the number of cfu within each cup could be determined. Cups which contained rabbit antiserum and mycoplasma organisms were sampled in the same manner. As shown in Table 3 there was a correlation between growth of the organisms and the colour changes produced. On an arbitrary basis we had previously chosen the end-point for the serum titre as the dilution of serum which prevented a colour change of greater than 50 % when compared with the same dilution of organisms grown in the absence of antiserum. It may be seen that at the serum end-point there was at least a tenfold suppression of growth of the three mycoplasmas tested. In addition, it is clear that the organisms must grow within the cups to at least 10^6 cfu/ml. in order to produce a colour change of half a pH unit.

Effect of number of organisms used in the test on the titre of rabbit antiserum

The FH strain of *M. pneumoniae* was tested with pre-inoculation and post-inoculation rabbit sera which had not been previously heat-inactivated. A 'block' titration was performed by diluting the organism suspension in serial tenfold steps and the sera in serial twofold steps. Plate 1 shows the test on the tenth day of incubation at 34° C. when progressive colour changes had ceased to occur. The organism grew to a titre of about 10^6 ccu, so that cups which contained the 10^{-5} dilution of the organism suspension were inoculated with about 10 ccu. At this dilution the pre-inoculation rabbit serum had a reciprocal titre of 10 and the post-inoculation serum a titre of 5120 or greater. With the 10^{-2} dilution (inoculum of 10^4 ccu) the pre-inoculation serum had a reciprocal titre of less than 10 and the post-inoculation serum a titre of 640. On the fourth day, however, when the 10^{-2} dilution had just produced a pH change of half a unit the titre of the post-inoculation serum with this dilution of organisms was 5120 or greater.

'Block' titrations of organisms and antisera of the type shown in Plate 1 were undertaken on several occasions with both *M. pneumoniae* and *M. fermentans*. The results of these kinetic studies are shown in Table 4. The serum titre was relatively unaffected by the size of the inoculum in the test if serum titres were recorded at a time when the pH of the medium in the control titration of the inoculum had just changed half a pH unit. Therefore, when a large number of sera were to be tested, multiple dilutions of organisms were not used; the test was performed with a

mycoplasma dilution which contained 10^3–10^4 ccu and was read when the pH of the medium containing this dilution had just changed half a pH unit.

Effect of heating sera

In the experiment shown in Plate 1, the pre-inoculation rabbit serum at a dilution of 1/10 inhibited the colour change produced by *M. pneumoniae*. Heating the serum at 56° C. for 30 min. abolished this slight inhibitory effect and did not significantly alter the titre of post-inoculation sera, as shown in Table 5. Therefore, in other tests heat-inactivated sera were used.

Table 4. *Effect of size of mycoplasma inoculum used in the test on the growth-inhibition titre of specific rabbit antisera*

Size of inoculum (no. of ccu/ 0·05 ml.)	Growth-inhibition titres* (Rabbit antiserum and homologous organism)								
	M. pneumoniae					*M. fermentans*†			
	Expt.					Expt.			
	1	2	3	4	5‡	1	2	3	4
$10^{5.5}$	NT§	1280	NT	NT	NT	2560	5120 or >	2560	NT
$10^{4.5}$	5120	2560	5120	2560	2560	2560	5120	2560	640
$10^{3.5}$	2560	2560	NT	5120	5120	1280	2560	640	NT
$10^{2.5}$	5120	2560	5120	2560	NT	1280	2560	NT	1280
$10^{1.5}$	2560	NT	NT	NT	5120 or >	2560	NT	NT	NT

* Titres determined at a time when the medium containing the indicated inoculum had just changed half a pH unit.

† Tests with *M. fermentans* performed with the addition of 6% unheated guinea-pig serum.

‡ Expt. 5 performed with the 1428 strain of *M. pneumoniae*. All other expts. with the FH strain.

§ Not tested.

Table 5. *Titre of unheated and heat-inactivated rabbit antisera in FI tests with homologous mycoplasma organisms*

Treatment of antiserum	Mycoplasma used in test and serum titre obtained			
	M. pneumoniae		*M. fermentans*	
	Test 1	Test 2	Test 1	Test 2
Unheated	5120	5120	5120	2560
Heated at 56° C. for 30 min.	5120	5120	2560	2560

Effect of heating the horse-serum component of the medium

When the horse-serum component of the medium was heated at 56° C. for 1 hr., the ability of *M. pneumoniae* antiserum to inhibit the growth of its homologous organism was lost. This finding indicated that a heat-labile factor in horse serum was required as a co-factor for inhibition of growth of *M. pneumoniae* by specific

antibody. The use of medium heated at 34° C. for 3 days or stored at 4° C. for 2 weeks, did not affect the growth-inhibition titre of *M. pneumoniae* rabbit antiserum.

Effect of unheated and heated guinea-pig serum (GPS)

The result of the experiment described above suggested that the labile factor necessary to demonstrate the growth inhibition of specific antiserum might be complement or a complement-like substance. The possibility that the addition of GPS to the reaction mixture might enhance the titre of rabbit antiserum was investigated. Ten per cent GPS, either unheated or heated at 56° C. for 30 min., in the standard medium with additives was added in the final 0·125 ml. so that the concentration of GPS in each reservoir was approximately 6 %. As shown in Table 6, the growth of the FH strain of *M. pneumoniae* was decreased 10,000-fold or more by unheated GPS while heated GPS decreased growth 100-fold. Similar results were obtained with the 1428 strain of *M. pneumoniae*. This inhibition of growth was a reproducible phenomenon. However, the FI titre of the rabbit antiserum was unaffected by the addition of either heated or unheated GPS.

Table 6. *Effect of guinea-pig serum (GPS) on mycoplasma growth and on the titre of homologous antiserum in FI tests*

		Growth suppression* (ccu) by		FI titre of homologous rabbit antiserum after addition to the test† of		
Expt. no.	Mycoplasma tested	Unheated GPS	Heated GPS	Medium alone	Unheated GPS	Heated GPS
1	*M. pneumoniae* strain FH	$> 10^4$	10^2	5120	2560	5120
2	*M. fermentans*	$10^{0.5}$	NT‡	5120 or >	2560	NT
3	Negroni agent	$> 10^2$	NT	< 320	2560	< 320
4	Negroni agent	10^5	$10^{0.5}$	320	5120	NT

* Suppression of growth in the presence of 6 % final concentration of GPS compared with growth in the absence of GPS.

† 0·025 ml. antiserum + 0·05 ml. mycoplasma + 0·125 ml. standard medium, either alone or containing 10 % GPS; therefore, final concentration of GPS about 6 %.

‡ Not tested.

Unheated GPS either delayed the colour change produced by *M. fermentans* at any particular dilution of the organism or, at the most, decreased the growth titre by less than tenfold. The FI titre of the *M. fermentans* rabbit antiserum was not increased by the addition of unheated guinea-pig serum to the test. However, the inhibitory effect of antiserum on fermentation produced by a high concentration of organisms was maintained for a longer interval when GPS was added to the reaction mixture than when it was omitted from the medium. In further tests with *M. fermentans* unheated GPS was, therefore, added to the medium. Unheated and heated GPS had an effect on the growth of the Negroni agent similar to that observed with *M. pneumoniae*. However, the FI titre of *M. pneumoniae* rabbit antiserum was not enhanced by the addition of unheated GPS to the test whereas the inhibitory titre of Negroni rabbit antiserum was markedly increased (Table 6).

Heated GPS did not produce a similar effect. Therefore, in further tests with the Negroni agent unheated GPS was used.

In some tests with the mycoplasma organisms and their homologous rabbit antisera it was observed that high dilutions of antiserum inhibited colour change whereas the lowest dilutions, i.e. those containing the highest concentrations of antibody, did not inhibit the colour change. With *M. fermentans* the addition of unheated GPS to the medium suppressed this colour change in cups which contained a high concentration of specific antiserum.

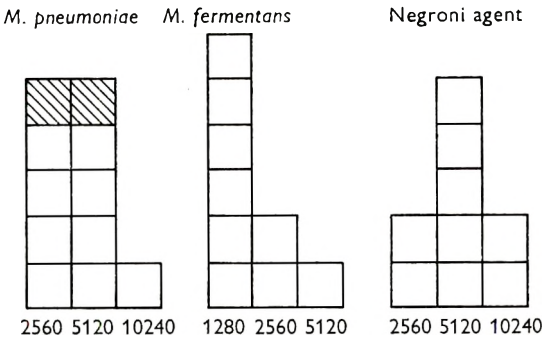

Fig. 1. Rabbit antiserum titres obtained in FI tests with homologous mycoplasma organisms, *M. pneumoniae*, *M. fermentans* and the Negroni agent. Each block represents the serum titre obtained in one test. ▨, 1428 strain; □, FH strain.

Table 7. *Results of cross FI tests*

Rabbit antiserum prepared to	Titre of serum in test with *M. pneumoniae* (FH strain)	*M. fermentans**	Negroni agent*
M. pneumoniae	2560	< 10	< 10
M. fermentans	< 10	1280	< 10
Negroni agent	< 10	< 10	5120
M. hominis type 1	< 10	< 10	< 10
type 2	< 10	< 10	< 10
M. salivarium	< 10	< 10	< 10
M. orale	< 10	< 10	< 10
Oral strain CH 20247	< 10	< 10	< 10
Navel	< 10	< 10	< 10

* Tests with this mycoplasma performed with the addition of 6% unheated guinea-pig serum.

Reproducibility

Figure 1 shows the results of a number of tests with *M. pneumoniae*, *M. fermentans* and the Negroni agent and their respective rabbit antisera which were performed at different times and by different persons. The same mycoplasma pool and the same hyperimmune serum was used for each test. The sera were initially diluted 1/2·5 or 1/5 and then diluted further in twofold steps by means of wire

loops. Only a two- to fourfold variation in serum FI titre was observed during the course of eleven titrations of *M. pneumoniae* antiserum, nine of *M. fermentans* and nine of Negroni agent antiserum.

Specificity

As shown in Table 7, rabbit antisera to *M. pneumoniae*, *M. fermentans* and the Negroni agent exhibited high homologous titres of growth-inhibiting antibody as measured by inhibition of colour change. On the other hand, these antisera did not inhibit the growth of the heterologous mycoplasma strains. In addition, rabbit antisera to *M. hominis* type 1, *M. hominis* type 2, *M. salivarium*, *M. orale*, the Navel strain of mycoplasma and a newly identified species from the human oropharynx, strain CH 20247 (Taylor-Robinson, Fox & Chanock, 1965), each at a dilution of 1/10, did not inhibit the growth of the three mycoplasma species studied. Although the homologous growth-inhibition titre of each of the last six antisera was unknown, they all possessed high levels of complement-fixing and indirect-haemagglutination antibodies and they all produced zones of inhibition in the disk growth-inhibition test (Taylor-Robinson *et al.* 1964; Taylor-Robinson, Fox & Chanock, 1965).

Table 8. *Antibody titres in paired sera from volunteers inoculated via the oropharynx with* Mycoplasma pneumoniae

	Reciprocal titres of pre- and post-inoculation sera with the indicated organism tested by the indicated technique									
	M. pneumoniae (FH strain)						*M. fermentans*, Negroni agent,			
	IHA		TRI		FI		FI		FI	
Volunteer										
To.	20*	1280†	< 5*	160†	< 5*	160†	< 5*	< 5†	< 5*	< 5†
Be.	20	1280	< 5	320	< 5	320	< 5	< 5	< 5	< 5
Bu.	< 10	40	< 5	160	< 5	160	< 5	< 5	< 5	< 5
Ha.	< 10	640	< 5	80	< 5	160	< 5	< 5	< 5	< 5
McM.	< 10	1280	< 5	640	< 5	320	< 5	< 5	< 5	< 5
Wi.	20	320	< 5	320	< 5	320	5	10	< 5	< 5
Fi.	40	160	< 5	320	< 5	640	< 5	< 5	< 5	< 5
Fe.	10	320	< 5	320	< 5	320	< 5	< 5	< 5	< 5
Va.	< 10	640	< 5	320	< 5	160	< 5	< 5	< 5	< 5
Jo.	320	640	< 5	160	< 5	160	< 5	< 5	< 5	< 5
Bo.	NT	NT	< 5	80	< 5	80	5	20	< 5	< 5
Sm.	NT	NT	< 5	2560	< 5	2560	< 5	< 5	< 5	< 5

* Pre-inoculation serum. † Post-inoculation serum.

Measurement of antibody in human sera

Adult volunteer sera tested against Mycoplasma pneumoniae. Sera were obtained from adult volunteers before and about 3 weeks after inoculation via the nasopharyngeal route with *M. pneumoniae*. They were tested by FI, TRI and IHA with the homologous organism and by FI with *M. fermentans* and the Negroni agent. The results are recorded in Table 8. All the tests with *M. pneumoniae* demonstrated fourfold or greater antibody rises, except IHA in which one of the paired

sera showed only a twofold rise. The results obtained in the FI and TRI tests were in very close agreement, indicating that the two tests were about equal in sensitivity. The occurrence of only one fourfold FI antibody rise to *M. fermentans* and the failure to detect a response to the Negroni agent further demonstrated the specificity of the technique.

Sera from healthy adults tested against Mycoplasma pneumoniae *and* M. fermentans. Sera from ninety-five adults, mostly 30–59 years of age and a few older, were tested. The usefulness of the technique as a means of detecting and measuring antibody in human sera is indicated by the results shown in Table 9. Twenty-two per cent of the sera contained FI antibody at a reciprocal titre of 5 or greater against *M. pneumoniae* and 13·5% contained antibody against *M. fermentans*. Because of the specificity of the test, these data suggest that such persons had been infected with these or very closely related mycoplasma species.

Table 9. *Results of FI tests with* Mycoplasma pneumoniae *and* M. fermentans *and ninety-five sera from adults without respiratory disease*

Organism used in test	No. of sera with reciprocal titre of:									% sera positive, titre 5 or >
	< 5	5	10	20	40	80	160	320	640 or >	
M. pneumoniae (1428 strain)	74	5	6	2	2	3	3	0	0	22
M. fermentans	82	4	2	4	2	0	0	0	1	13·5

DISCUSSION

M. pneumoniae and *M. fermentans* have been shown to ferment glucose probably to lactic acid and it is presumed that the colour change in the test described in this communication is the result of glucose fermentation. Because of this we have referred to the test as one of fermentation inhibition (FI). The biochemical basis for the production of acid by the Negroni agent has not been examined, so that for this and other acid-producing mycoplasmas the description of the test as one of fermentation inhibition may be premature. However, regardless of the mechanism of the acid production, the decrease in pH and consequent colour change of the indicator associated with growth of the organisms is inhibited by the addition of antiserum.

Several factors concerned in the performance of the test have been evaluated. Heat inactivation of sera is a worth-while procedure since it did not decrease the titre of antisera, and with *M. pneumoniae* it eliminated presumably non-specific inhibition produced by the pre-inoculation rabbit serum. The importance of using unheated horse serum in the growth medium cannot be overstressed since the specific inhibitory effect of antisera was markedly reduced when the organisms were grown in medium containing heat-inactivated horse serum. The inhibitory activity of rabbit antiserum was partially restored by the addition of 6% unheated GPS to the test. This indicates that a heat-labile accessory factor present in horse and guinea-pig serum is important for the demonstration of specific antibody activity; whether or not this is a heat-labile component of complement is

unknown. This observation is in agreement with that of Priestley (1952) who worked with *M. mycoides*, but contrary to that of Edward & Fitzgerald (1954) who studied human genital strains. It is possible that the requirement for a heat-labile accessory factor in the inhibition of growth of various mycoplasmas by specific antiserum varies with the mycoplasma studied. The effect of GPS on the growth of mycoplasmas and on the growth-inhibitory activity of their antisera is indicated by a consideration of the three mycoplasmas studied. The growth of *M. pneumoniae* was inhibited by both unheated and heated GPS. The addition of unheated GPS to the test did not enhance the growth-inhibitory activity of specific rabbit antiserum provided that unheated horse serum was present in the medium. The growth of *M. fermentans* was little affected by unheated GPS and the growth-inhibition titre of specific antiserum was not increased. Finally, with the Negroni agent, unheated GPS not only inhibited growth of the organism but was essential for demonstrating the effect of specific antiserum. Thus, the need for unheated horse serum in tests with *M. pneumoniae* and *M. fermentans* and the additional need for unheated GPS in tests with the Negroni agent indicate the varying requirements for a heat-labile accessory factor. The different requirements could reflect a qualitative difference in the factor present in horse or guinea-pig serum or a quantitative difference in requirement for the same factor in horse and guinea-pig serum. These problems will be solved only when the nature of the accessory factor has been determined.

It was demonstrated with *M. fermentans* that the addition of unheated GPS inhibited the 'break-through' of growth in cups which contained a high concentration of specific antiserum. Growth in the presence of a high concentration of antibody may be due to the growth-stimulating effect of serum. Partial and reversible growth inhibition of mycoplasmas in the presence of excess antibody has been observed by Bailey, Clark, Felts & Brown (1963). Regardless of the mechanism of this 'zoning' phenomenon, the fact that it occurs is a pitfall in the 'screening' of sera at low dilution. Growth-inhibiting antibody could be overlooked unless sera are diluted sufficiently and tested over a range of dilutions.

Rabbit antisera prepared against all the human mycoplasma species exhibit low levels of heterotypic cross-reactivity in CF and IHA tests (Taylor-Robinson *et al.* 1963; Taylor-Robinson *et al.* 1964; Taylor-Robinson, Fox & Chanock, 1965). The same sera, however, did not produce heterotypic growth inhibition of the organisms used in the present study; the test we describe therefore appears to be extremely specific. The growth-inhibition technique employing filter-paper disks soaked in antiserum (Huijsmans-Evers *et al.* 1956; Clyde, 1964) is also specific but not applicable to the quantitative measurement of antibody because of its low sensitivity. Although the FI technique has a less generalized application, since it can only be used for acid-producing mycoplasmas, it appears to be at least as sensitive as other serological techniques. Thus, the titres obtained for rabbit sera in FI tests with homologous organisms were as high as the titres obtained previously with the same sera in CF and IHA tests. In addition, various levels of growth-inhibitory antibody to *M. pneumoniae* and *M. fermentans* have been found in random sera from human adults without respiratory illness. The antibody response of adult

volunteers to infection with *M. pneumoniae* measured by FI was not different from the response measured by TRI, indicating that the two techniques have about equal sensitivity.

With the use of standard pools of organisms, the results obtained have been very reproducible. This was true even with the use of widely varying doses of organisms provided that serum titres were recorded at a time when the concentration of organisms used in the test had just produced a colour change of half a pH unit. The fact that a standard mycoplasma inoculum was not essential increased the simplicity of the test even more. This simplicity is in contrast to the laborious nature of the immunofluorescence technique and some other growth-inhibition methods (Bailey *et al.* 1961; Jensen, 1963).

A test which measures growth-inhibiting antibody to mycoplasmas has various applications. First, it should be useful in assessing the immunological status of individuals before experimental or natural infection. Measurement of growth-inhibiting antibody may be a better index of protective immunity than measurement of other antibodies, i.e. CF, IHA. Secondly, the test may be used as a tool in epidemiological studies. In this connexion, the occurrence of antibody to *M. fermentans* in 13·5 % of adult sera diluted 1/5 is of interest since reports of the isolation of this mycoplasma species from man are few (Huijsmans-Evers & Ruys, 1956; Ruiter & Wentholt, 1953). Recently, 'virus-like' particles associated with human leukaemic bone marrow (Murphy, Furtado & Plata, 1965) have been identified as *M. fermentans* in this laboratory. In addition to *M. fermentans* and the Negroni agent, other acid-producing mycoplasmas have been recovered recently in association with human leukaemia and human tumours (Mittelman, Horoszewicz & Grace, 1965). Finally, therefore, the test provides not only an opportunity to study the interrelationship of these acid-producing mycoplasmas but also an opportunity to assess their significance in relation to human disease.

SUMMARY

A fermentation-inhibition test for the measurement of growth-inhibiting antibody to acid-producing mycoplasmas was performed in microtitre plastic plates. *M. pneumoniae*, *M. fermentans* and the Negroni agent were selected for study. Antibody could be titrated since specific antiserum inhibited mycoplasma growth and the concomitant production of acid, thus preventing a change in colour of phenol red which was incorporated in the growth medium. It was essential to use unheated horse serum as a component of the growth medium. The inhibitory effect of specific antiserum was much decreased when the horse serum was heat-inactivated, indicating the need for a heat-labile accessory factor. The additional use of unheated guinea-pig serum was essential for demonstrating the growth-inhibiting effect of specific antiserum on the Negroni agent. The test was reproducible, specific and sensitive. Sixteen-fold or greater antibody rises were demonstrated in paired sera from volunteers infected with *M. pneumoniae*. Tests with ninety-five random adult sera showed that 22 % had antibody to *M. pneumoniae* at a titre of 1/5 or more and 13·5 % had antibody to *M. fermentans*.

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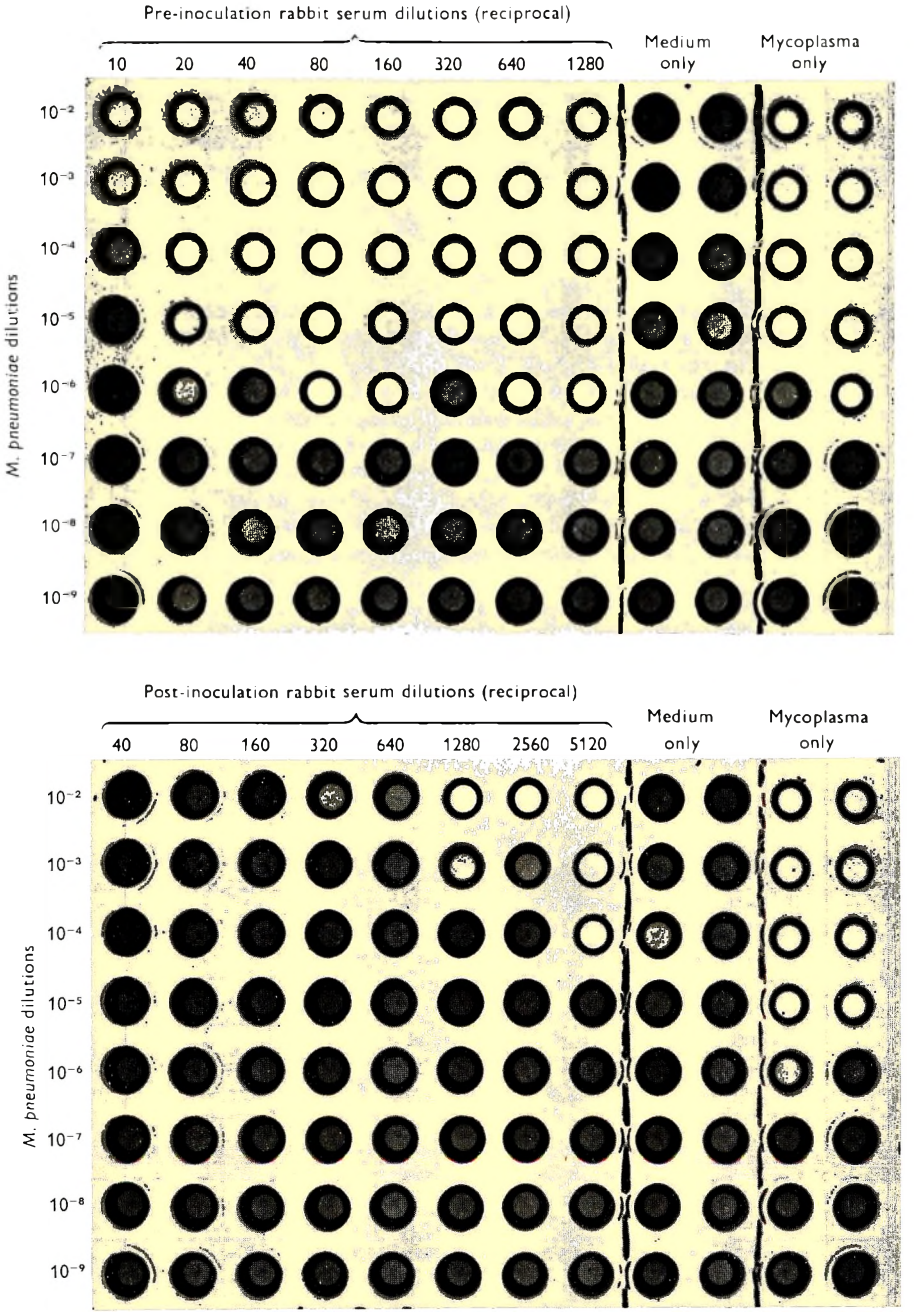
EXPLANATION OF PLATE

'Block' titrations in disposable microtitre plates of *Mycoplasma pneumoniae* with pre-inoculation and post-inoculation rabbit sera. Cups which appear dark contain medium of pink colour (pH 7·8); cups which appear colourless contain medium of green-yellow colour (pH < 6·5). Photograph taken on the tenth day of incubation at 34° C. At 10^{-5} dilution of mycoplasma the titre of the post-inoculation serum is 5120 or greater.


Pre-inoculation rabbit serum dilutions (reciprocal)
10
20
40
80
160
320
640
1280
Medium only
Mycoplasma only
M. pneumoniae dilutions
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
10⁻⁸
10⁻⁹
Post-inoculation rabbit serum dilutions (reciprocal)
40
80
160
320
640
1280
2560
5120
Medium only
Mycoplasma only
M. pneumoniae dilutions
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
10⁻⁸
10⁻⁹

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A study of poliovaccination in infancy: excretion following challenge with live virus by children given killed or living poliovaccine

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INTRODUCTION

Infants in this country are usually vaccinated against diphtheria, tetanus, pertussis and poliomyelitis in the first year of life, and the resultant immunity is subsequently reinforced by booster doses at intervals.

It was the policy in the London County Council Clinics to offer immunization with a triple antigen (diphtheria, tetanus, pertussis) at age 2, 3 and 4 months, with a booster dose at 15 months. Immunization against poliomyelitis was carried out by three doses of trivalent oral poliovaccine at age 7, 8 and 9 months. When a quadruple vaccine, incorporating killed poliovaccine and triple antigen, became available (Beale & Ungar, 1962), it was decided to assess its value under the routine L.C.C. conditions. It was appreciated that the children might be too young for an optimal response to the poliovaccine component, since Perkins, Yetts & Gaisford (1958, 1959) have shown that the response, particularly to the type 1 component of an inactivated vaccine, was poor in infants under 6 months of age. They showed that the response was improved by increasing the strength of the type 1 component. We therefore used a vaccine containing an increased amount of type 1 poliovirus antigen.

The trial was designed with two main objectives. First, the serological response of infants to the poliovaccine component of quadruple vaccine was compared with the response of infants to oral poliovaccine as routinely administered. Second, the effect of administering graded doses of type 1 attenuated poliovirus was studied to assess the degree of gut immunity conferred by poliovaccination.

In order to keep the trial small enough to be feasible only a single type of poliovirus could be used for the challenge experiments. Type 1 virus was chosen since it is the most important type causing paralysis and because it has been more difficult to produce adequate immunity to this type with killed virus vaccine.

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PLAN OF THE TRIAL

The children were enlisted at the age of 2 months from two areas of London, each of which contributed about twenty-five children to each of the four groups, A, B, C and D. The groups received vaccine, and specimens of blood and faeces were examined in accordance with the schedules shown in Table 1.

Table 1. *Plan of trial*

Group	Vaccine	Surveillance	Blood sample	Challenge
A	Quadruple vaccine at 2, 3, 4 months	Monthly faeces specimens 2–6 months	At 2 and 6 months	At 6 months. Faeces twice weekly for 3 weeks after challenge
B	Triple antigen at 2, 3, 4 months	Monthly faeces specimens 2–6 months	At 6 months	At 6 months. Faeces twice weekly for 3 weeks after challenge
C	Primary quadruple vaccine 2, 3, 4 months. Booster at 15 months	Monthly faeces specimens 2–16 months	At 15 and 16 months (before and 1 month after booster)	At 16 months. Faeces twice weekly for 3 weeks after challenge
D	Primary triple antigen at 2, 3, 4 months. Booster at 15 months. Trivalent oral poliovaccine 7, 8, 9 months	Monthly faeces specimens 2–16 months	At 16 months (7 months after oral poliovaccine)	At 16 months. Faeces twice weekly for 3 weeks after challenge

The children in group A received a primary course of killed poliovaccine, those in group B constituted a control group who had no immunization against poliomyelitis. The children in group C received a primary course and a booster dose of killed poliovaccine, those in group D were immunized with oral poliovaccine. No detailed study of reactions to the vaccines was made, but reactions to quadruple vaccine did not seem to be more severe than those following triple antigen.

The children were randomly allocated to their vaccine groups and to five subgroups for challenge. The challenge doses consisted of five serial tenfold dilutions of attenuated type 1 poliovirus, LSc.2ab, the highest dose containing $10^{5\cdot7}$ TCD_{50}. Specimens of faeces were examined at monthly intervals from the start of the trial to challenge, so as to detect natural poliovirus infections, and twice weekly after challenge to estimate the excretion of the poliovirus type 1 challenge virus.

METHOD AND MATERIALS

Vaccines

The quadruple and triple vaccines and the attenuated oral poliovaccine were prepared by Glaxo Laboratories Ltd.; the quadruple vaccine was 'Quadrilin' batch 12, containing at least 75, 1 and 1 D antigen units of poliovirus antigen for types 1, 2 and 3 respectively, 28 Lf of diphtheria toxoid, 10 Lf of tetanus toxoid and 200 million killed *Bordetella pertussis* organisms in each 1 ml. dose. The triple antigen (batch 431) contained the same quantity of diphtheria, tetanus and pertussis antigens as the quadruple vaccine in each 0·5 ml. dose.

The trivalent oral attenuated polio vaccine was T.V. 101 prepared from the Sabin strains of attenuated poliovirus, containing $10^{5\cdot7}$ TCD_{50} of type 1 (LSc.2ab), $10^{5\cdot0}$ TCD_{50} of type 2 (Ch.2ab) and $10^{5\cdot5}TCD_{50}$ of type 3 (Leon.12ab) strains.

Challenge doses

The attenuated type 1 challenge virus was prepared from monovalent Sabin attenuated poliovaccine, Glaxo batch A.V. 5 type 1, and was preserved in 50% sucrose solution. Virus was diluted to the chosen strength and dispensed into dropper bottles, one for each child. Each bottle contained a dropper into which one dose could be drawn. The highest dose contained $10^{5\cdot7}TCD_{50}$, and there were four serial tenfold dilutions of this dose. The challenge doses at each dilution were checked for virus content before dispensing and were stored at $-25°$ C. until the day before they were required. After transport at ambient temperature, they were stored at 4° C. overnight. After a dose had been given, the bottle containing residual fluid was returned to the laboratory and stored at $-25°$ C. until it was retitrated. A random selection of each of the doses given at any clinic session was titrated, and the results are shown in Table 2. There was no difference between the titre of the challenge doses used early in the trial, i.e. for groups A and B, and those used 15–18 months later for groups C and D. The virus proved stable even in a diluted form when stored in 50% sucrose.

Neutralizing antibody titrations

These were performed against all three types of poliovirus by a micromethod as only small quantities of blood were obtained from the children by heel prick. The technique was based on that described by Durand (1962). Serial twofold dilutions of serum were made and mixed with an equal volume of poliovirus containing approximately 100 TCD_{50}. Virus-serum mixtures were held at 37° C. for 3 hr. before addition of cells. Virus-serum and cells were drawn up into capillary tubes, which were sealed with liquid paraffin and incubated at 37° C. The British standard poliovirus antisera were put up with each test so that the results could be expressed in terms of international units of antibody per ml. (Perkins & Evans, 1959; Lyng & Bentzon, 1963). The micromethod of performing the antibody titrations gave results similar to those obtained at Glaxo Laboratories with the cytopathic test used in the collaborative assay of the British Standard poliomyelitis antisera (Perkins & Evans, 1959).

Virus isolation

Surveillance and pre-challenge specimens of faeces were examined in secondary monkey kidney cells (Mair & Tobin, 1960) and in a continuous cell line (HeLa or HEp 2). Pre-challenge specimens from children who failed to excrete poliovirus type 1 after challenge were also examined in unweaned mice. Post-challenge specimens were examined only in monkey kidney cells.

Table 2. *The challenge doses of attenuated poliovirus type* 1

Dose	Dose at time of dispensing (TCD_{50})	After challenge		
		No. of titrations	Mean TCD_{50}/dose	Range
1	$10^{5.7}$	19	$10^{5.55}$	$10^{4.86-5.98}$
2	$10^{4.7}$	19	$10^{4.58}$	$10^{4.0-5.04}$
3	$10^{3.7}$	19	$10^{3.55}$	$10^{3.0-3.98}$
4	$10^{2.7}$	13	$10^{2.56}$	$10^{2.2-2.78}$
5	$10^{1.7}$	16	$10^{1.61}$	$10^{1.2-1.81}$

RESULTS

A total of 236 children entered the trial. Most of them were between 2 and 3 months of age, but thirty-six were just over 3 months old.

Twenty children failed to complete the appropriate course for the following reasons: moved away, 8; intercurrent illness, 5; irregular attendance, 5; given wrong vaccine, 1; severe reaction to vaccine, 1. Of those who completed the course 55 were in group A, 52 in group B, 52 in group C and 57 in group D.

Poliovirus antibody response

The antibody titres for the children in the four groups are shown in Fig. 1. (The presentation of the results has been simplified by assigning intermediate readings to the next lowest value in the twofold dilution series.) Owing to the small quantities of serum available for examination, many titrations did not give an end-point and an arrow shows that the titre was more or less than the indicated titre. The number of sera examined against each type of poliovirus is less than the number of children completing the course because some children could not be bled at the correct time, some specimens of serum were so small that they could only be tested against two virus types and some children experienced natural poliovirus infections. These last children were only excluded so far as the antibodies homotypic to their infecting virus were concerned since the response of these children to the other virus types was not different from that of uninfected children.

At the age of 2 months (group A, 1st serum) many children had high titres of maternal antibody, particularly to type 2 virus, and these antibodies had not entirely disappeared when the children were bled at 6 months of age (group B). In one area where records were available, 80 % of the mothers had been immunized —60 % with inactivated vaccine and 20 % with oral poliovaccine—either before or during the pregnancy resulting in the study child.

The primary course of quadruple vaccine (group A) stimulated the production of

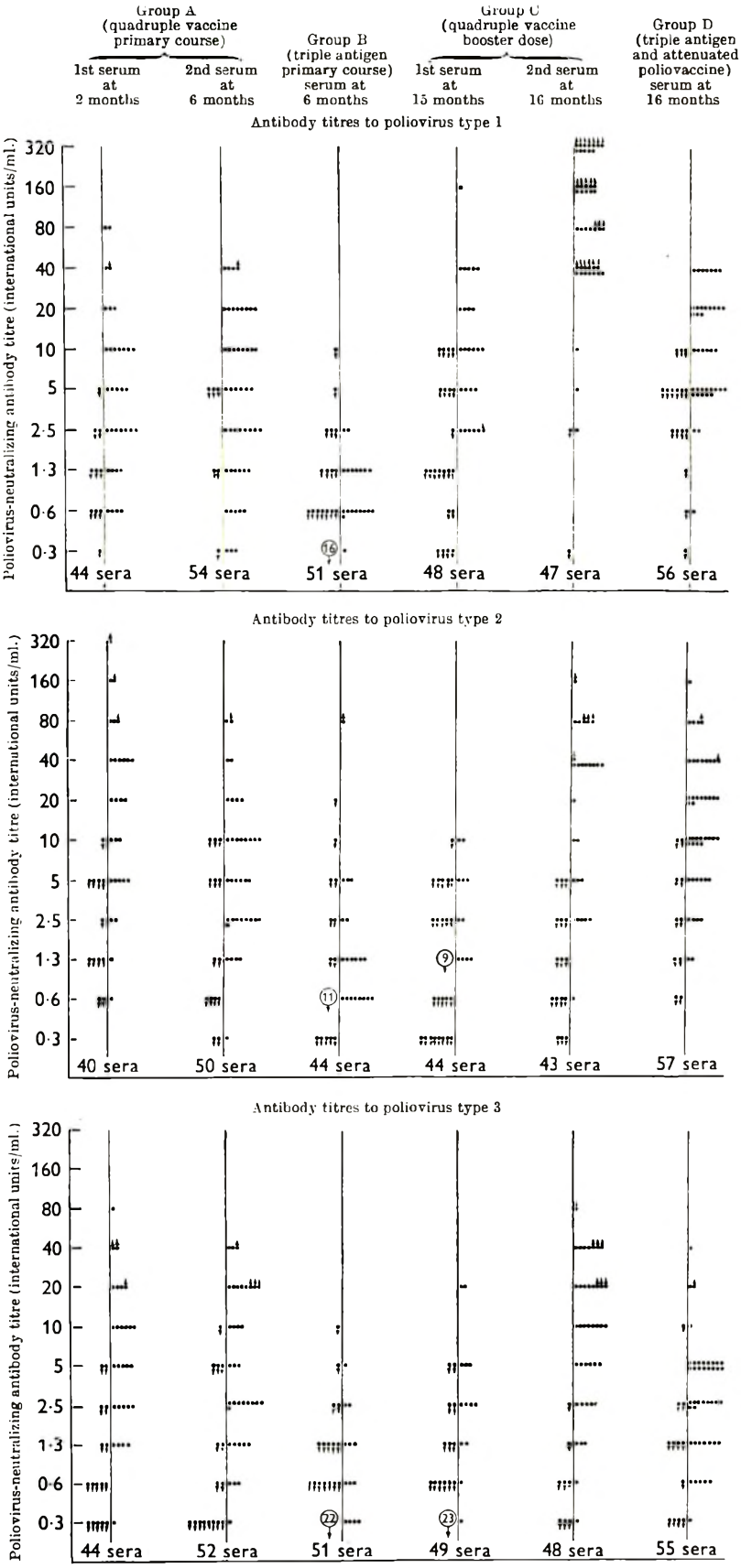
Group A (quadruple vaccine primary course)
1st serum at 2 months
2nd serum at 6 months
Group B (triple antigen primary course) serum at 6 months
Group C (quadruple vaccine booster dose)
1st serum at 15 months
2nd serum at 16 months
Group D (triple antigen and attenuated poliovaccine) serum at 16 months
Antibody titres to poliovirus type 1
Poliovirus-neutralizing antibody titre (international units/ml.)
320
160
80
40
20
10
5
2·5
1·3
0·6
0·3
44 sera
54 sera
51 sera
48 sera
47 sera
56 sera
Antibody titres to poliovirus type 2
40 sera
50 sera
44 sera
44 sera
43 sera
57 sera
Antibody titres to poliovirus type 3
44 sera
52 sera
51 sera
49 sera
48 sera
55 sera

Fig. 1.

some poliovirus antibodies which can be judged by comparing, in Fig. 1, the results for group A second sera with the group B children who had had no poliovaccine. A more precise analysis can be attempted by comparing the titres obtained from the sera of individual children before and after the primary course. The decline of maternal antibody has to be offset against active antibody production by the infant. Assuming that maternal antibody has a half-life of 28 days, its titre might be expected to fall 16-fold between the 2nd and 6th months of life. The following arbitrary categories have been defined:

Good response. Second titre equal to or greater than the first.

Doubtful response. Second titre half to one-eighth of first titre.

No detectable response. Second titre one-sixteenth or less of the first titre. A few children were included in this group when the second serum showed no antibodies when tested at a low dilution.

Unassessed. Insufficient data to assess response: few, if any, in this group made a good response.

Table 3. *Response to the primary course of inactivated vaccine*

	Number of children				
Poliovirus type	Good response	Doubtful response	No detectable response	Unassessed	Total
1	21	10	8	4	43
2	10	9	14	2	35
3	17	5	15	2	39

The response of the children to the primary course of vaccine is given in Table 3. Examination of the individual protocols showed clearly that it was those children with the lowest titres of maternal antibody who made the best response. Technically, of course, it is easier to demonstrate the active production of antibody in a child whose initial titre is low but the striking response of children with no initial antibody could not be explained in this way.

When the children in group C were 15 months of age, they were bled immediately before the booster dose. The antibody titres in the serum at this time were for the most part low. Some children, with no detectable antibody, reacted to one or more of the poliovirus antigens in the booster dose by producing antibodies to a high titre. In these children the antibody-forming mechanism had been sensitized by the primary course. The response to any one of the three poliovirus antigens was not related to the response to the other types.

Most children responded well to the booster dose of vaccine. A consideration of the titres reached after the booster dose offers a more realistic assessment of the immunization procedure than a comparison of the titre obtained from individual children before and after the booster dose. In assessing the response of the children to the complete course of vaccine, the following arbitrary definitions have been made:

Good response. Final serum titre equal to or greater than 10 I.U./ml.

Some response. Final titre 2·5 or 5·0 I.U./ml.

Minimal or no response. Final titre less than 2·5 I.U./ml.

For type 3 vaccine, half the above-mentioned units of antibody have been used.

The results obtained from the children in group C are given in Table 4. The response of a child to a course of poliovaccine is essentially individual and will depend, among many other things, on the amount of antigen injected and the titre of maternal antibody at the start of the course. The antibody titre after the booster dose is the most reliable indication of the effectiveness of the immunization procedure and a close study of the results obtained with the different virus types reveals interesting discrepancies when the assessment of the effectiveness of immunization is made at other times as shown in Fig. 1.

Table 4. *Serological response to the complete course of inactivated vaccine*

(Results assessed 1 month after booster.)

	Number of children				
Poliovirus type	Good response (≥ 10 I.U.)	Some response (2·5 or 5 I.U.)	Minimal or no response (< 2·5 I.U.)	No detectable antibody*	Total
1	43	2	2	0	47
2	18	8	14	3	43
3	30†	8†	9†	1	48

* Sera not tested at low dilution.
† Antibody levels one half those for types 1 and 2.

Table 5. *Serological response to the complete course of attenuated poliovaccine*

(Results assessed 7 months after third dose.)

	No. of children with antibody titre				
Poliovirus type	Good response (≥ 10 I.U.)	Some response (2·5 or 5 I.U.)	Minimal or no response (< 2·5 I.U.)	No detectable antibody*	Total
1	24	15	8	9	56
2	35	10	8	4	57
3	20†	17†	15†	3	55

* Sera not tested at low dilution.
† Antibody levels one half those for types 1 and 2.

The serological results from the children in group D who were bled 7 months after their last dose of live vaccine are given in Fig. 1 and Table 5. It will be seen that the circulating antibody titres, especially for type 1, were not as high as they were 1 month after a booster dose of killed poliovaccine, but it may be that the titre of antibody is not the most important criterion of immunity after oral poliovaccine.

Infection after challenge

Challenge doses were given to 216 children. In determining whether or not a child became infected as a result of the challenge dose, twenty-six children had to be excluded: natural infection with poliovirus type 1 during surveillance, 7;

concurrent infection with another virus at challenge, 15; too few faeces specimens examined after challenge, 4.

The results of feeding graded doses of Sabin's type 1 vaccine virus to 190 children are shown in Table 6.

It will be seen that the smallest challenge dose (about 50 TCD_{50}) was capable of infecting about half the children in groups A and B and that a primary course of killed poliovaccine did not influence the size of dose required to infect. The children in group D, who had had attenuated oral vaccine, required a much higher dose to initiate infection. The children in group C seemed a little more resistant to infection than those in groups A and B, but a proportion could still be infected by the lowest challenge dose. Infection after challenge was not influenced by the titre of neutralizing antibody at the time, nor was it affected by natural infection with poliovirus types 2 or 3 during surveillance.

Table 6. *Excretion of poliovirus type* 1 *after challenge*

		Vaccine group			
Dose sub-group	Dose (TCD_{50})	A (quadruple vaccine, primary course)	B (triple antigen)	C (quadruple vaccine, after booster)	D (triple antigen, attenuated poliovaccine)
1	$10^{5.7}$	9/10	9/9	8/8	8/11
2	$10^{4.7}$	9/9	9/10	9/9	7/12
3	$10^{3.7}$	10/11	11/11	4/8	1/10
4	$10^{2.7}$	7/9	6/8	4/10	0/8
5	$10^{1.7}$	7/10	5/10	3/8	0/9
Total		42/49	40/48	28/43	16/50

Numerator = no. of excretors; denominator = no. challenged.

Duration of excretion

It was possible to assess whether or not challenge led to infection in 190 children. In considering the duration of excretion of poliovirus type 1 after challenge, it was necessary to exclude a further 13 children; 2 of these became infected with another virus during the observation period and 11 (4 in group A, 4 in group B and 3 in group C) did not submit a specimen after the 16th day, poliovirus having been isolated from their last specimen.

The duration of excretion of the remaining 177 children is given in Table 7. Specimens of faeces were examined twice weekly, and the duration of infection has been calculated as half-way between the last positive and the first negative specimen.

It will be seen that almost all the children in group B who became infected excreted poliovirus type 1 for more than 18 days after challenge. About two-thirds of the infected children in group A and one half of those in group C excreted for this period. None of the infected children in group D excreted for more than 14 days. Provided that infection was initiated, the size of the challenge dose had no effect on the duration of excretion.

Poliovirus type 1 was isolated on more than one occasion from all the children in groups A, B and C who were infected after challenge, but from seven of the children in group D it was isolated from a single specimen only. However, the timing of these positive specimens and the titre of virus they contained indicated that virus multiplication had occurred in the gut.

Table 7. *Duration of excretion of poliovirus type* 1 *after challenge*

	Number of children in vaccine group			
Duration of excretion (days)	A (quadruple vaccine, primary course)	B (triple antigen)	C (quadruple vaccine, after-booster)	D (triple antigen, attenuated poliovaccine)
No excretion	7	8	15	34
1–9	1	0	0	5
10–18	13	2	12	10
> 18	23	34	13	0
Total children	44	44	40	49

Table 8. *Effect of pre-challenge antibody titres on duration of excretion after challenge*

Duration of excretion (days)	No. of children with indicated serum titre after primary course quadruple vaccine (A)		No. of children with indicated serum titre after booster dose of quadruple vaccine (C)	
	< 10 I.U.	≥ 10 I.U.	< 100 I.U.	≥ 100 I.U.
No excretion	3	4	3	12
1–9	0	1	0	0
10–18	6	7	2	10
> 18	18	5	7	5
Total	27	17	12	27

Effect of antibody titre on duration of excretion

In order to determine whether the child's antibody titre at the time of challenge had any effect on the duration of the subsequent poliovirus excretion, the children in groups A and C were divided according to their antibody titre, with the results shown in Table 8. One child in group C has been excluded since no serum was obtained.

It will be seen that in group A those children with the higher antibody titres tended to excrete poliovirus for a limited period only. A similar trend can be discerned among the group C children, although the actual titres of antibody were much higher. In this group there appears to be an excess of children with high antibody titres who were not infected; it is difficult to assess the significance of this finding, since by chance these children all received the smaller doses of challenge virus.

In group D, fifteen children excreted poliovirus after challenge. The duration of this excretion did not appear to be related to the child's antibody titre at the time of challenge.

Amount of virus excreted after challenge

Poliovirus type 1 was excreted by 126 children after challenge. All the faecal extracts from which poliovirus had been isolated were titrated using tenfold dilutions and two tubes per dilution. The 50 % end-point was calculated and plotted graphically against the day after challenge on which the specimen had been collected. The area under the curve for each child was measured with a planimeter; from the result the mean virus content of the faeces was calculated from day 6 to day 15 after challenge and expressed as log TCD_{50} per gramme faeces per day.

Table 9. *Excretion of poliovirus* 6–15 *days after challenge*

Vaccine group	Number of children with mean log TCD_{50}/day/g. faeces				Total
	< 3·0	3·1–4·0	4·1–5·0	> 5·0	
Primary quadruple vaccine (A)	5	11	20	4	40
Primary triple antigen (B)	0	2	20	16	38
Booster quadruple vaccine (C)	2	8	11	2	23
Attenuated poliovaccine (D)	12	2	0	0	14
					115

The results obtained from eleven children have had to be excluded for the following reasons: intercurrent infection with another virus, 2 children; faeces extracts toxic, 3 children; too few specimens during the relevant period, 5 children; excretion for less than 6 days, 1 child. The results for the remaining 115 children are given in Table 9.

From the 6th to the 15th day after challenge, most of the unimmunized children in group B excreted more than 10^4 TCD_{50} poliovirus/g. faeces per day, and nearly half of them excreted more than 10^5 TCD_{50}. Few of the children in groups A and C excreted 10^5 TCD_{50}/g. faeces per day and about 40 % of the children in these two groups excreted less than 10^4 TCD_{50}. Twelve of the fourteen infected children in group D excreted less than 10^3 TCD_{50}/g. faeces per day.

The period 6–15 days after challenge was chosen for the calculation, since the largest number of observations was available for plotting the excretion curve for each child. Similar results were obtained when the virus excreted for the period 10–19 days after challenge was calculated, but naturally there was a marked shift to the left; 36 % of the children in group A, half of those in group C and all of those in group D excreted 10^3 TCD_{50}/g. faeces per day or less. Only one child in group B came into this category.

If infected faeces are the means whereby poliovirus spreads in a community, and if a child is to pass on his infection to others, this must happen while the virus titre in the faeces is reasonably high. If it is assumed that children ingest milligrams

rather than grams of faeces then a virus titre in the faeces of the order of 10^4 TCD_{50}/g. is necessary for a child to be infective. Clearly, the amount of faeces passing from child to child will depend on the domestic hygiene of the family; under present-day conditions the above assumption appears justified.

Figure 2 shows how the different vaccine schedules affected the potential infectivity of the children. All children have been included, whether infected or not, who received the four highest challenge doses of poliovirus and who had not

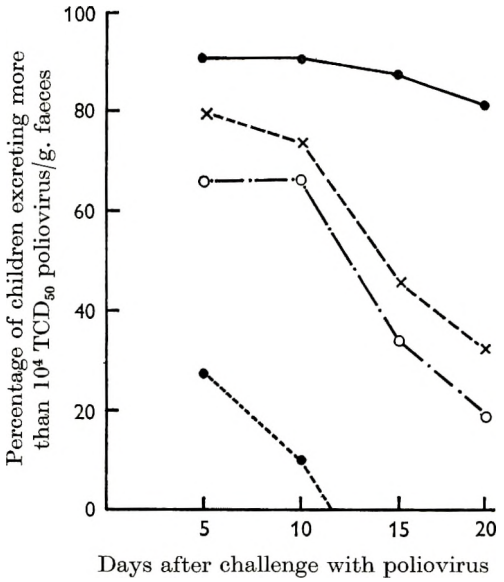

Fig. 2. Effect of vaccination schedules on potential infectivity of children. ●—●, Group B, triple antigen. × — — ×, Group A, primary course, quadruple vaccine. ○—·—○, Group C, primary and booster dose, quadruple vaccine. ● - - - - ●, Group D, attenuated poliovaccine.

Table 10. *Effect of pre-challenge serum titre on amount of virus excreted 6–15 days after challenge*

Vaccine group	Serum titre (I.U.)	No. of children with mean log TCD_{50}/day/g. faeces				
		< 3·0	3·1–4·0	4·1–5·0	> 5·0	Total
Primary course quadruple vaccine (A)	< 10	1	7	15	3	26
	⩾ 10	4	4	4	1	13
Booster dose quadruple vaccine (C)	< 100	0	1	4	2	7
	⩾ 100	2	7	6	0	15

been excluded from any previous analysis. The proportion excreting 10^4 TCD_{50}/g. faeces at different times after the challenge dose has been plotted. The area under the curves gives a measure of the infectivity of groups of children vaccinated in different ways and exposed to infection with at least 500 TCD_{50} of attenuated virus. When the areas were measured, it was found that, taking the infectivity

of the unvaccinated as 100, the infectivity of the children who had had killed vaccine was 60 while that of the children immunized with oral vaccine was about 10. These observations were confined to the first 20 days after infection; it seems probable that had the observations been continued, the differences between the groups would have been greater.

Influence of antibody on amount of virus excreted

If the results obtained from the children in groups A and C are divided according to the antibody titre in the pre-challenge specimen of serum, Table 10 is obtained. Two children have been excluded, since no specimens of their sera were examined. In both groups the children with the higher antibody titres tended to excrete less poliovirus than those who had responded less well to the vaccine.

DISCUSSION

Serological results

Killed poliovaccine

The striking feature of the results when antibody titres at 16 months are studied is the excellent response to the type 1 component of the quadruple vaccine compared with that to types 2 and 3. This was presumably due to the high D-antigen content of the type 1 component (Beale & Ungar, 1962); similar good results have been obtained in older children by Dane *et al.* (1962). The inhibitory effect of maternal antibody, first reported by Perkins *et al.* (1958, 1959), was overcome by the potent type 1 antigen.

The results at 15 months (Fig. 1 group C, first serum) show in general the expected fall in antibodies after the primary course and titres were disappointingly low in some children, nevertheless the response to the booster dose showed that the children without detectable antibodies fell into two groups. One group failed to respond to the primary course, probably because of inhibition by maternal antibody, and the other group had been sensitized and made a booster response. Some children in group C (10 out of 48) had antibody titres to type 1 virus of 20 units or more at this time—a figure not expected from the results given by the children in group A after the primary course. These high titres might have been caused by missed natural poliovirus infections but this suggestion was not supported by the results of challenge with attenuated virus. Butler, Barr & Glenny (1954) using diphtheria toxoid found that the response of infants who lacked maternal antibody was excellent. They also observed that, in those with maternal antibody, the antibody response was delayed so that when infants were immunized at 1 and 6 weeks of age and bled at 3 months and again at 6 months, titres of diphtheria antitoxin were higher at the second bleeding. A similar delay in poliovirus antibody production may have operated in this trial.

The response to poliovirus types 2 and 3 was less good than that to type 1. Quadruple vaccine of the same composition as used in this trial was found to give excellent antibody titres for types 2 and 3 in children over 6 months of age (Dane *et al.* 1962) and also in children whose immunization was started at age 4 months

(Dane, 1964). The discrepancy between these results and our findings could well be due to the earlier start of immunization in this study and to the high titres of maternal antibody due to the policy of active immunization of the mothers. A delay of 2 months will be associated with a fourfold fall in the titre of maternal antibody, assuming as we have done a half-life of 28 days, based on the work of Orlandini *et al.* (1955). The antibody titres were assessed only 1 month after the booster dose, and the duration of the high titres has not been ascertained. We have found however (A. J. Beale, unpublished) that the antibodies produced by this course of quadruple vaccine are 7S immunoglobulins (1 gG) and are therefore likely to be associated with a long-lasting immunity.

Attenuated oral poliovaccine

The serological results for the children who had attenuated poliovaccine are at first sight disappointing; some children had little or no detectable antibody even though they had received a course of immunization generally accepted as adequate. The children in group D were bled 7 months after their third dose of poliovaccine. It was found in the Public Health Laboratory Service trial of live poliovaccine (Report, 1961) that, except possibly for type 3, antibody titres did not change much between 1 and 6 months after the third dose of a course of oral poliovaccine. The distribution of antibody titres of all three types reported here did not differ greatly from the results obtained from the sera examined at Colindale in the P.H.L.S. trial; comparative titrations of antibody carried out by Glaxo Laboratories and the Virus Reference Laboratory, Colindale, have in the past given similar results (Perkins & Evans, 1959).

The titres observed in the children who responded to the killed vaccine (group C) were much higher for types 1 and 3 than those in the children who had had attenuated vaccine. The possibility that this might represent a strain-specific response was considered. Some sera were retested using attenuated viruses rather than our stock strains (Brunenders type 1, MEF 1 type 2 and Saukett type 3) but the difference between groups C and D could not be explained in this way.

The serological response may not be the most important criterion of immunity after oral poliovaccine. The excretion of poliovirus by the children in group D after challenge showed that there was a substantial degree of intestinal immunity which was unrelated to the titre of circulating antibody. Moreover almost all the children with no detectable antibody (whose sera could be tested at low dilutions) were known from the monthly surveillance specimens to have excreted virus of the appropriate type during the time that they were receiving attenuated vaccine.

Infection following the challenge dose of poliovirus

It was not surprising that it was possible to infect unimmunized infants with a dose of poliovirus of about thirty virus particles (equivalent to 50 TCD_{50}) since the virus dose involved in the transmission of the natural infection is probably of this order. The children who had received a primary course of killed poliovaccine were just as susceptible to infection with small doses of challenge virus as the

unimmunized controls. Children who had been given a primary course of killed vaccine were also susceptible to natural infection with poliovirus; the fifty-two children in group C experienced thirteen natural infections with poliovirus between the end of their primary course and their booster dose 11 months later. Four of these infections were with type 1, seven with type 2 and two with type 3.

The children in group C, challenged 1 month after their booster dose of quadruple vaccine, were somewhat more resistant than the children in groups A and B to infection with challenge doses of 5000 TCD_{50} or less. Calculation, from Table 6, shows that, in order to infect 50 % of the children in group C, a dose of virus 8–10 times larger was needed compared with groups A and B. These children were older and although age by itself may not increase the dose required to infect, it implies that the children had been exposed to the risk of natural infection for longer and this might not have been detected by the examination of the monthly surveillance specimen. Any failure to detect natural infection would, of course, make the children in group C appear more resistant to infection. The serological results and the excretion of virus after challenge did not suggest that we failed to detect natural infection in many children. It is not possible to say whether the increased resistance to infection was a function of the increased age of the children or the result of their active immunization.

As was expected, the children in group D were considerably more resistant to infection with the challenge virus, requiring about 1000-fold higher doses to infect them compared with the unimmunized controls.

Duration of excretion and amount of virus excreted

The quantitative study of virus excretion after infection showed some interesting differences between the various groups. Most of the unimmunized children in group B excreted large amounts of virus for the whole of the 3-weeks observation period. The children who had had the killed vaccine, groups A and C, showed a tendency to excrete less virus than the unimmunized controls. This tendency was most obvious in the children who had responded best to the killed vaccine by producing the higher antibody titres.

The effect of the serum antibody titre on the duration and amount of poliovirus excreted was clearly not a direct one. As can be seen from Tables 8 and 10 the children in group A with antibody titres of 10 I.U./ml. or more showed a limitation of virus excretion similar to that of the children in group C with titres of 100 I.U./ml. Those children in group D who were infected by the challenge dose excreted only small amounts of virus for short periods, yet their serum antibody titres at the time of challenge were only about one-tenth of those in group C. It is not known how a natural poliovirus infection of the intestine is brought to an end; possibly antibody is produced in the gut in response to the presence of virus there. This antibody-producing mechanism might be partially sensitized by parenteral injections of killed vaccine and come into play more rapidly after infection.

A study by the P.H.L.S. (Report 1965) also showed some reduction in poliovirus excretion by children who had received a primary course of killed vaccine, but the

conditions of this study were so different from ours that detailed comparison is not possible.

In agreement with these findings Galbraith (1964) observed that fewer Salk-vaccinated children were excreting poliovirus in a random survey compared with unvaccinated children of the same age. This phenomenon may afford an explanation for the greater protection of communities by killed poliovaccine than would be expected from the number vaccinated. This appears to have occurred in Sweden, where only killed poliovaccine has been used (Gard, 1964), and also in Holland according to a report by Hofman in a paper presented at a meeting of the Society for General Microbiology in 1964. Gard reports that in Sweden no cases of clinical poliomyelitis have occurred since 1963 in either the vaccinated or unvaccinated section of the population. He also reported that no isolations of poliovirus had been made from individuals admitted to infectious disease wards of hospitals from 1963 onwards although other enteroviruses remained prevalent.

The community effect following the widespread use of attenuated poliovaccine is well known. It is difficult to infect children immunized in this way except with large doses of virus, and their infectivity, compared with unvaccinated controls, is reduced by 90 %. Children immunized with killed vaccine can still be infected with quite small doses of virus and their infectivity is reduced only by 40 %. A smaller community effect may be expected from the use of killed vaccines.

SUMMARY

Quadruple vaccine containing 75 D antigen units of killed type 1 poliovirus was given to children at ages 2, 3 and 4 months followed by a booster dose at 15 months.

The serological response to the primary course was difficult to assess owing to maternal antibody. Antibody titres to the type 1 component after the booster dose were very satisfactory and about 10 times higher than those observed in a similar group of children given attenuated vaccine. Response to the poliovirus types 2 and 3 in the quadruple vaccine was less satisfactory.

Graded doses of attenuated poliovirus type 1 were fed to the children 2 months after the primary course and 1 month after the booster dose. Children who had received no poliovaccine and children immunized with attenuated vaccine were included for comparison.

Immunization with killed vaccine did not greatly affect the size of the minimal infecting dose of live virus but reduced both the duration of the subsequent infection and the titre of virus in the faeces.

The epidemiological significance of these findings is discussed.

A trial of this type which involved the collection of nearly 3000 specimens of faeces could not have been completed without the help of a large number of people. We are particularly indebted to Dr W. G. Harding and to Dr F. R. Waldron in whose Divisions the trial was carried out, to Miss Conway and to Mrs Watson who were responsible for the record-keeping and co-ordination in the two

Divisions, and to the Centre Superintendents and Health Visitors on whom the continued contact with the children depended. We are also most grateful to the mothers of the children for their regular attendance at the special clinic sessions, for sending the faecal specimens and for allowing us to investigate the response of their children to vaccination.

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Source of *Pseudomonas aeruginosa* infection in premature infants

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In January 1965 two babies died in the premature-baby ward of the Queen Victoria Memorial Hospital, Melbourne, from respiratory infection due to *Pseudomonas aeruginosa*. An investigation was promptly commenced in an attempt to confine this potentially serious outbreak.

Most reports of outbreaks caused by Gram-negative bacilli in premature-baby wards have indicated that oxygen equipment, mechanical mucus aspirators (Rogers, 1960; Becker, 1962; Kresky, 1964; Bassett, Thompson & Page, 1965) or the humidifying water of incubators (Sever, 1959; Foley, Gravelle, Englehard & Chin, 1961) are the most likely reservoirs of infection. Other sources of these organisms are water taps, faucet aerators and sink traps. For example, Wilson, Nelson, Phillips & Boak (1961) found *Ps. aeruginosa* in four out of five aerators sampled and Kresky (1964) reported that the same organism persisted in these sites in spite of frequent cleaning. Cabrera & Davis (1961) dramatically terminated an outbreak of *Flavobacterium* meningitis by repair of a leaking sink trap which harboured the organism.

In the study reported here the nursery equipment, wash basins and air conditioners were extensively sampled for *Ps. aeruginosa* and other Gram-negative bacilli. From this emerged a clear picture of heavy contamination of suction equipment and its connecting tubes with a variety of strains of *Ps. aeruginosa*, some of which were similar to those concerned in the fatal infections. Not only was it apparent that infants could be infected by the introduction of contaminated catheters into their respiratory tracts, but it was also demonstrated that the exhaust outlets from the aspirator jars were capable of discharging large numbers of organisms into the ward as an aerosol spray. This report re-emphasizes the importance of contaminated suction equipment as a source of *Ps. aeruginosa* infections and describes how aerial dispersion of this organism was controlled by modifying the design of the suction unit.

EPIDEMIOLOGY

The ground plan of the premature-baby ward (Fig. 1) shows seven bays, separated by 8 ft. partitions, an isolation room and service areas. The smallest babies are nursed in bays 1 and 2, containing respectively four and six incubators ('Insul-cots', The Commonwealth Industrial Gases Limited) similar to the type

shown in Plate 1*a*. As the infants thrive they are moved progressively through the bays in numerical sequence.

Five babies became infected with *Ps. aeruginosa* in January 1965. Three cases presented initially with eye infections and the other two with infections of the nose and throat. Case 1 had been transferred from bay 2 to the isolation room in

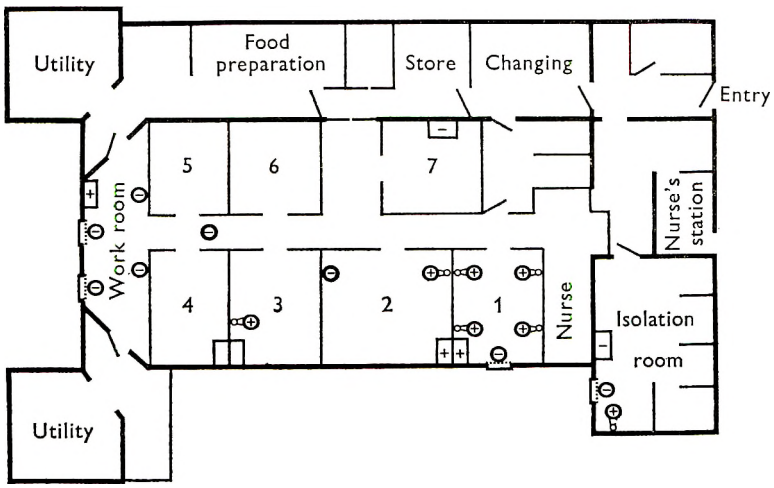

Fig. 1. Plan of premature-baby ward with subdivision into bays, isolation room and other service areas.

December, when an eye swab yielded heavy growth of 'coliforms'. Cases 2, 3 and 4 were infected in bay 1, where they were nursed until 22 January when case 2 died at 16 days from a suppurative pneumonia and case 3 at 9 days from a lung abscess. After these deaths, the gravity of the situation was realized and case 4 was immediately transferred to the isolation room. One week later case 5 (a carrier detected during routine swabbing of all babies in the ward) was also removed to the isolation room.

BACTERIOLOGICAL INVESTIGATIONS

Various sites in the ward and isolation room were sampled, sometimes on several occasions, with large swabs moistened in nutrient broth. These were plated directly on nutrient agar, horse-blood agar and MacConkey's agar and were also inoculated into nutrient broth. All cultures were incubated aerobically for 24–48 hr. at 37° C. Swabs taken from inside surfaces of air conditioners, window ledges and crevices were, in addition, cultured in cooked-meat medium in a search for anaerobic organisms.

Water (20–50 ml.) from humidifying reservoirs in the incubators was passed through 'Millipore' membranes which were cultured on nutrient agar. Samples of aqueous 'Zephiran' solutions containing aspirated mucus were also filtered through membranes which were then washed with distilled water and cultured in nutrient broth containing 10% 'Lubrol W'.

Air entering the nursery was sampled by exposing agar plates for 10 min. against the grilles of the air conditioners and also by culturing glycerol-moistened gauze strips which had been held in the same position for 24 hr. Nursery air was sampled in the central corridor between bays 4 and 5, using a Casella slit air sampler. The gas discharge from suction units was directed on to horse-blood agar plates for 5 min.

Ps. aeruginosa was identified by morphology, colony characteristics and pigment production on nutrient agar and King's medium (King, Ward & Raney, 1954). An attempt was made, in the absence of pyocines and specific typing sera, to determine whether the strains were identical or dissimilar by testing their sensitivity to a number of bacteriophages. Their antibiotic sensitivity was also investigated.

RESULTS

These are summarized in Table 1, which shows the distribution of *Ps. aeruginosa* in the premature-baby ward. It is evident that this organism was found consistently in the suction tubes, and to a lesser extent in the wash-basin outlets. The suction tubes were also contaminated with other Gram-negative bacilli, including *Klebsiella*, and occasionally with yeasts.

In view of the heavy contamination of the suction tubing, other parts of the 'Twin-O-Vac' suction units (The Commonwealth Industrial Gases Limited) were examined. These were mounted on the dividing partitions and walls and were connected to a piped oxygen supply which delivered oxygen to the intranasal catheter via a water bubbler and, by means of a venturi system, effected suction of mucus from the mouth into a glass jar containing aqueous 'Zephiran' 1 in 1600. Since the inlet tube to the jar did not extend below the disinfectant solution, aspirated material could be expelled directly to the ward. Table 2 records the results of all samplings.

Ps. aeruginosa was isolated regularly from the rubber suction tubing and metal inlets to the jar. Two of the six outlet tubes through which the exhaust air was discharged also harboured the organism at the time of sampling. Impingement plate cultures of exhaust air and oxygen from one of four units tested yielded a heavy growth of *Ps. aeruginosa* and other organisms as shown in Plate 1*b*.

We were not in a position to type the strains by the pyocine or serological methods described by Wahba (1965), but they were tested with bacteriophages available in this department (Holloway, Egan & Monk, 1960). The results, shown in Table 3, indicate that the strains could be separated into six different phage patterns, labelled for convenience A–F, and that the two fatal cases were due to dissimilar organisms. Some strains from the suction tubes corresponded to the strains causing infections, but those from the sink outlets were of unrelated patterns.

All the strains were sensitive to polymyxin, and moderately sensitive to tetracyline and chloramphenicol. Three were distinguished by resistance to streptomycin; these were in phage-group C (Table 3) and were unrelated to the strains causing the infections.

Table 1. *Distribution of* Pseudomonas aeruginosa *in premature-baby ward*

Source of cultures	Location	No. examined	No. positive for *Ps. aeruginosa*	No. positive for other Gram-negative bacilli
Suction tubes	Bay 1	4	4	4 (+ yeasts)
	Bay 2	2	2	2 (+ yeasts)
	Bay 3	1	1	1 (*Klebsiella*)
	Isolation room	1	1	1 (+ yeasts)
Oxygen tubes	Bay 1	4	0	0
	Isolation room	1	0	0
Oxygen bubblers	Isolation room	2	0	1
Incubator walls and ports	Bay 1	1	0	0
	Isolation room	1	0	0
Incubator water	Bay 1	3	0	0
	Isolation room	2	0	1
'Zephiran' solution (suction jars)	Bay 1	1	0	1
	Isolation room	1	0	1
Wash-basin outlets	Bay 1	1	1	1
	Bay 2	1	1	1
	Bay 7	1	0	1
	Isolation room	1	0	1
	Workroom	1	1	1
Taps and spray	As above	5	0	0
Air conditioners	Bay 1	1	0	0
	Isolation room	1	0	0
	Workroom	2	0	0*
Ward air	Corridor	1	0	0†

* *Cl. perfringens* and other *Clostridium* species isolated.
† 6 colonies/c.ft. (organisms unidentified).

Table 2. *Contamination of suction units with* Pseudomonas aeruginosa

Location of Unit	Rubber tubing		Jar inlet	Jar outlet	Exhaust oxygen
	Proximal*	Distal†			
Bay 1 Unit 1	+	+	+	+	−
Unit 2	+	+	+	−	+
Unit 3	+	+	+	−	−
Bay 2 Unit 1	+	+	+	−	N.D.
Unit 2	+	+	+	−	N.D.
Isolation room	+	+	+	+	−

* The end joined to the oral catheter.
† The end connected to the inlet tube of suction jar.
N.D. = test not done.

Table 3. *Phage grouping of* Pseudomonas aeruginosa *strains isolated from premature-baby ward*

Pattern	Phages E79	Phages F116	Phages 120X	Phages 2X	Number of isolates	Source of strains
A	−	+	−	+	10	Case 5 5 suction units (bays 1 and 2) Oxygen exhaust (bay 1)
B	+	+	−	+	7	Cases 3 and 4 Suction unit (isolation room)
C	+	−	−	+	4	2 wash-basin outlets (bay 1 and workroom) 2 suction units (bays 1 and 2)
D	+	+	+	+	3	3 suction units (bays 1 and 2)
E	+	−	+	+	1	Case 2
F	+	−	−	−	1	Wash-basin outlet (bay 3)

MODIFICATION OF THE SUCTION EQUIPMENT

It was obvious that the suction tubes and aspirator jars were the most likely sources of cross-infection in this nursery, the transmission being effected either by gravity flow into the oral catheter, which was connected to the pressure tubing by a simple glass bulb, or by aerial dispersion in the exhaust from the suction jar. The former mechanism was easily prevented by connecting the catheter to a glass bulb fitted with an internally sealed tube to act as a 'no-return' trap for the mucus secretions and replacing the rubber tubing by clear disposable plastic which could be inspected for cleanliness and dryness. The cost of a 4 ft. length was approximately one shilling.

To prevent aerial dispersion from the suction jar a modification of the unit was required, namely the introduction of a liquid trap and fibrous filter in the unit. In order to test the efficiency of various filters and liquid traps an apparatus, illustrated diagrammatically in Fig. 2, was set up.

In this system an atomized culture of *Ps. aeruginosa* was introduced into an aerosol mixing chamber, from which the aerosol was drawn for 7 min. through the suction bottle and then into an exposure chamber. The difference in bacterial counts on nutrient agar plates from the aerosol and exposure chambers gave a measure of the efficiency of filters and liquid traps, either singly or in combination.

Table 4 shows results which are typical of many tests performed with this apparatus. It will be seen that the high count of airborne bacteria was progressively reduced, first by the glass-wool filter, more effectively by the water trap and completely by a combination of both.

As a result of these experiments the design of the 'Twin-O-Vac' unit was altered. The conversion of the standard to modified unit consisted of extending the inlet tube close to the bottom of the jar and attaching to the exhaust outlet a 3 in. metal filter packed lightly with glass wool (Corning Glass Co., no. 28C). Aqueous

chlorhexidine (300–400 ml. of 1 in 10,000) is used as a bacteriostatic liquid trap and is changed at least once a week. The standard and modified units are illustrated in Plate 2*a*, *b*.

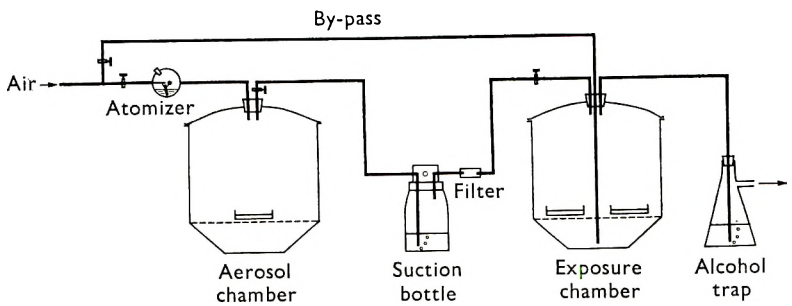

Fig. 2. Diagram of apparatus used for testing fluid traps and fibrous filters attached to suction jar.

Table 4. *The efficiency of removal of bacterial aerosols by modified suction equipment*

Suction equipment	Colony counts* of *Ps. aeruginosa* from Aerosol chamber	Exposure chamber
Standard	Uncountable	Uncountable
Modified with glass-wool filter	Uncountable	300–400
Modified with water trap (400 ml.)	Uncountable	17
Modified with glass-wool filter and water trap	Uncountable	0

* Nutrient agar plates were exposed during passage of aerosol through the apparatus for 7 min.

DISCUSSION

It is well known that *Ps. aeruginosa* survives and multiplies in moist conditions and this study has shown that the lumen of rubber suction tubes provided a consistent source of infection (Table 1). The wash-basin outlets yielded three isolations out of five samplings, but none of these strains corresponded to those associated with the infections (Table 3). The taps, including a spray device, the humidifying water in the incubators and the rubber oxygen tubes were all negative for *Ps. aeruginosa*, although one oxygen bubbler and the water from one cot reservoir produced heavy growth of other organisms. It seemed evident that the suction units and their tubing provided the main source of infection in this ward, a finding similar to that of Bassett *et al.* (1965). These authors abandoned the use of aspirators, parts of which are difficult to sterilize, in favour of disposable or boilable mucus extractors.

Apart from the obvious role of contaminated mucus gravitating into the oral catheter and thus initiating a contact infection, there exists also the problem of its dispersion as an aerosol spray in the ward. This could lead to the establishment of

widespread foci in the ward or nursery environment. For various reasons connected with the nursing of the babies in the hospital concerned, it was decided to continue the use of mechanical suction with the following recommendations:

(1) The standard 'Twin-O-Vac' unit was replaced with one possessing a liquid trap and a glass-wool or similar fibrous filter.

(2) Clear disposable plastic tubing was substituted for rubber tubing and was replaced daily.

(3) A glass bulb trap was used to prevent gravitational flow of mucus from the suction tubing to the oral catheter.

(4) The catheter and glass bulb were autoclaved before use.

(5) The suction jar was dismantled and cleaned weekly and recharged with 300–400 ml. of 1 in 10,000 aqueous chlorhexidine.

Since the introduction of these precautions no further infections due to *Ps. aeruginosa* have occurred, and swabs from the plastic tubing have yielded negative cultures.

SUMMARY

1. A small but serious outbreak of *Ps. aeruginosa* infection in a premature baby ward has been described.

2. Heavy contamination of the suction apparatus and tubing was demonstrated to be the main reservoir of infection.

3. It was also shown that this apparatus could disseminate the organism as an aerosol.

4. The measures adopted to eliminate the source and prevent dissemination of the infectious agent have been described.

We wish to thank Dr A. Wheildon, superintendent of the Queen Victoria Memorial Hospital, and the nursing sisters in the premature-baby ward for most helpful collaboration, also Mr H. Berkshire, the clinical photographer at the hospital, and officers of The Commonwealth Industrial Gases Limited. Dr B. W. Holloway of this Department assisted in phage-grouping the *Pseudomonas* cultures. One of us (J.C.F.) is supported by a grant from the National Health and Medical Research Council.

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EXPLANATION OF PLATES

PLATE 1

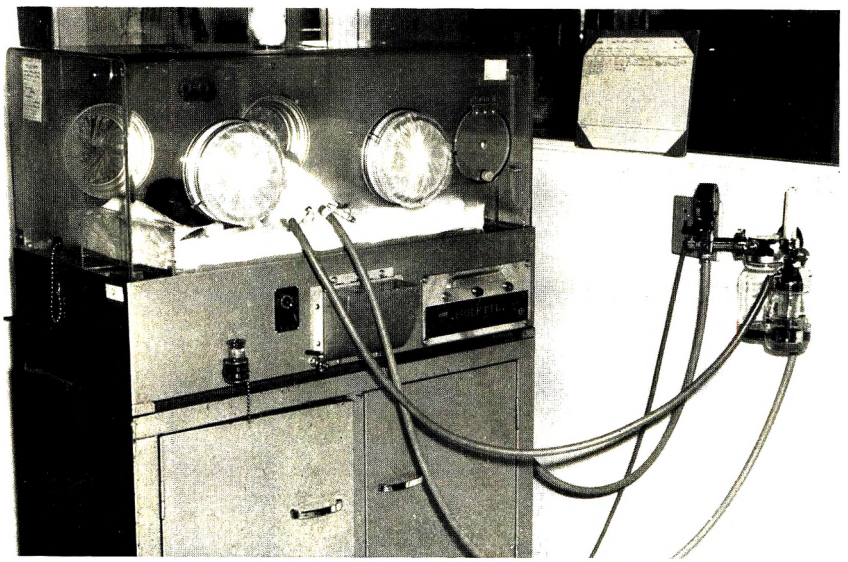
(*a*) Incubator ('Insul-cot' or 'Isolette') showing wall-mounted suction units ('Twin-O-Vac') for delivery of oxygen and removal of mucus by suction. Note the connexion between unit and incubator is by rubber tubing.

(*b*) Horse-blood agar plate exposed to exhaust oxygen from suction jar for 5 min. Twenty of the colonies were *Ps. aeruginosa*.

PLATE 2

(*a*) Standard 'Twin-O-Vac' suction unit showing oxygen humidifier and suction jar with rubber tubing connexion to the incubator.

(*b*) Modified unit showing liquid trap, glass-wool filter and clear plastic tubing.



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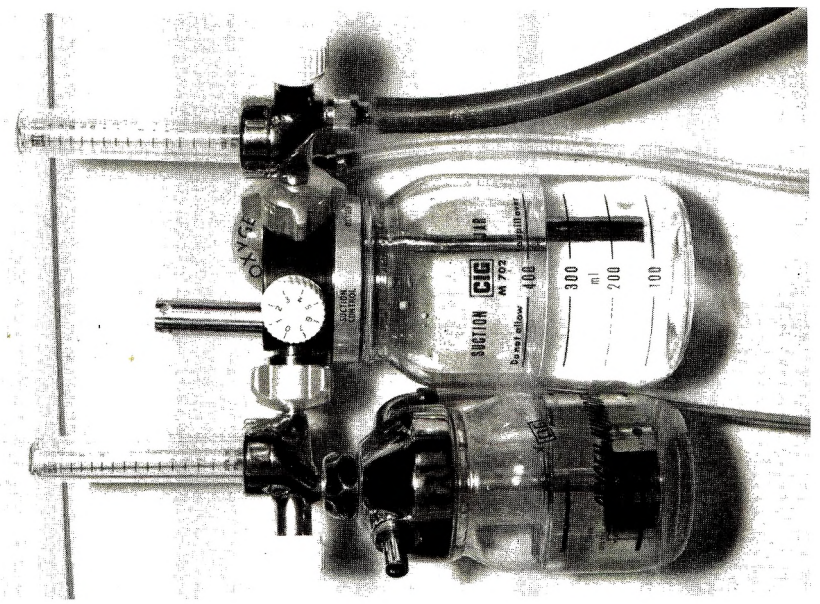
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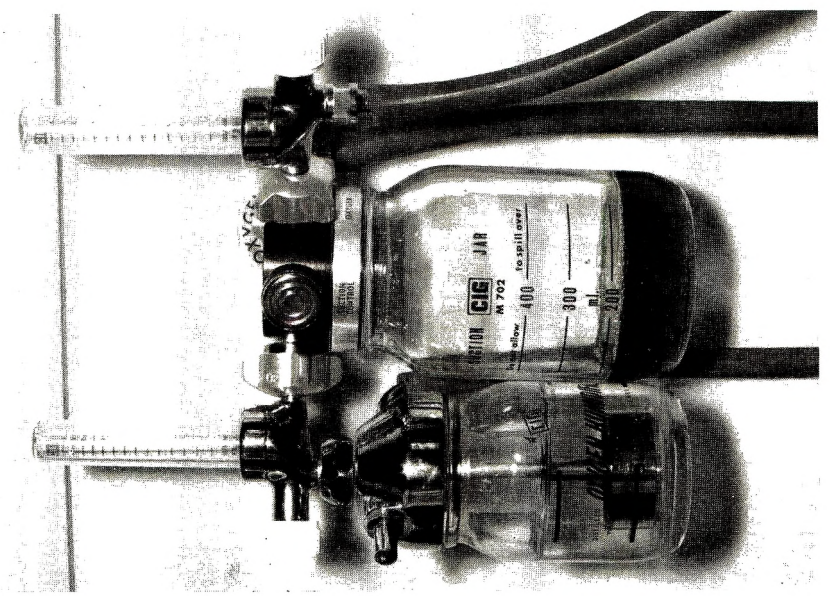
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