J. Hyg., Camb. (1964), **62**, 401
Printed in Great Britain

A complement fixation technique for the quantitative measurement of antigenic differences between strains of the virus of foot-and-mouth disease

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(*Received* 30 *January* 1964)

The early literature on the specificity of the immunological types of foot-and-mouth disease virus included references to minor antigenic differences which occurred between strains of virus of the same type. Such strains were called variants. The value of these observations, however, was limited by the techniques then available for differentiation. Bedson, Maitland & Burbury (1927) applied an *in vitro* serum neutralization test in their comparison of two strains of virus of Type A. They recorded results which indicated that higher serum neutralization titres were obtained with homologous serum–virus mixtures than with heterologous mixtures.

Progress in this field of investigation resulted from the development of the complement fixation test. Traub & Möhlmann (1946) used this test to demonstrate antigenic differences between three strains of virus of Type A and also drew attention to the possible significance of these observations in relation to the efficiency of protection by vaccines. Subsequent work at this Institute was directed to the correlation of the results obtained by cross-vaccination, cross-serum neutralization and cross-complement fixation tests. In the first of these experiments Galloway, Henderson & Brooksby (1948) compared three strains of virus of Type A, A119 (A_{12} Pirbright) with MP and M1 from the Mexican outbreak of 1947–49. The comparison of a further ten pairs of strains of Type A, Type O, Type SAT 1 and Type SAT 2 (Rice & Brooksby, 1953; Brooksby, 1952; Davie, 1962; Martin, Davies & Smith, 1962; Hyslop, Davie & Carter, 1963; and unpublished work) has confirmed that there are strains of virus of the same immunological type which can be classified as antigenic variants or subtypes within the types and that in general there is good correlation between the results of the three tests. The results of the two serological tests and the cross-vaccination tests showed that three of the ten pairs were of the same subtype and that seven of them were dissimilar.

It has been found that the reproducibility of the results obtained in the complement fixation test and its convenience are of advantage when a large number of strains of virus of one type have to be examined. The complement fixation test was applied by Traub & Möhlmann (1946) to demonstrate differences in the titre of immune sera reacting with homologous and heterologous antigens. Brooksby, Galloway & Henderson (1948) used the amount of complement fixed as a measure of such reactions, while Graves (1960) combined the titration of complement with the titration of antisera in order to compare homologous and heterologous antigen–

antibody combination. Bradish, Brooksby & Tsubahara (1960) and Bradish & Brooksby (1960) made a detailed examination of the reactions of foot-and-mouth disease antigens and antisera in the complement fixation test and established certain principles for the comparison of virus strains. They demonstrated that the amount of complement fixed by the reaction of an antigen with an antiserum was in proportion to the concentration of antibody when the antigen was present in limited excess in the reacting mixture; that a comparison of the amounts of complement fixed by two or more strain-specific antisera in an excess of one antigen allowed a more sensitive detection of differences between strains of virus than when two antigens and one antiserum were used; and that, if two or more strain-specific antisera were titrated with one preparation of antigen, the reactions of each mixture of antigen and antibody throughout the dilution series could be measured as the amount of complement fixed at optimum proportions of antibody with limited excess of antigen and compared as the cross-fixation ratio of each serum to that of the homologous mixture. When the cross-fixation ratios of a range of strain-specific antisera were compared in their reactions with the virus strains used to prepare the sera, the relationship of one virus strain to the other could be expressed as the product of their cross-fixation ratios one with the other. Two virus strains were distinct if the cross-fixation product was significantly different from unity.

MATERIALS AND METHODS

A series of virus strains of type O was examined, including the established subtypes O_1, O_2 and O_3. The other virus strains included nine strains isolated from field outbreaks of disease in Britain from 1957 to 1961 and several other strains of virus maintained at Pirbright for experimental purposes. It was not known whether any of these strains had a common origin.

The virus antigens and antisera used in the tests were prepared in guinea-pigs. Original virus samples recovered from cattle were adapted to growth in guinea-pigs by intradermal inoculation of the metatarsal pad and serial passage of infected pad material. A virus was considered to be adapted to the guinea-pig when secondary lesions developed on the metacarpal pads or tongue following metatarsal pad inoculation, usually at the third to sixth passage. The guinea-pigs used for passage of the virus strains and the preparation of sera were kept in strict isolation, one room being set aside for each strain examined.

Antigens

The antigens were prepared from infected guinea-pig pad material. The pads and vesicle fluid were collected 24 hr. after intradermal inoculation of the metatarsal pads. Antigen suspensions were prepared by grinding one pad in 1 ml. of 0·04 M phosphate buffer, pH 7·6, to give a 1/10 suspension and clarified by centrifugation with a laboratory centrifuge at 3000 r.p.m. for 10 min. The suspensions were prepared immediately before the tests and the infected pads were used within 48 hr. of collection. The infectivity of these antigens was in the region of 10^8 mouse ID 50 per ml.

Antisera

Guinea-pig antisera were used in the tests. 600–800 g. guinea-pigs were inoculated intradermally with infected pad material of high titre and, if secondary lesions developed, the guinea-pigs were held for 4 weeks. They were then given two intramuscular injections of 1 ml. of a 1/5 suspension of fresh infected pads at an interval of 4 days. Ten days after the last inoculation, the guinea-pigs were bled and the serum collected and pooled. The pooled serum was filtered through a Seitz E.K. pad, inactivated at 56° C. for 30 min. and stored in convenient quantities at −20° C. The stored serum was diluted as described below before the tests and inactivated for a further 30 min. at 56° C.

The complement fixation test

This followed the basic procedure developed and described by Brooksby (1952) for the 'short' or 'routine' test. The complement used was normal guinea-pig serum freeze-dried and stored under vacuum. Immediately before each test the dried complement was reconstituted and diluted in veronal buffer with added calcium and magnesium. The dilution chosen, usually 1/20 or 1/25, was that at which a 0·1 ml. dose would give approximately 50 % haemolysis in the complement control titration. This standard dilution was found to be constant within each batch of dried complement. Subdilutions were prepared in which seven doses of complement in a logarithmic series of 0·09, 0·13, 0·2, 0·3, 0·45, 0·67 and 1·0 ml. were obtained when pipetting a standard 1·0 ml. amount of complement dilution into each tube. Five doses of complement within this series were chosen to cover the expected 50 % haemolytic end-point in each set of five tubes containing the reacting mixtures of antigen and antiserum or control titrations of the reactants alone.

The haemolytic indicator system consisted of a 1·5 % suspension of washed sheep cells sensitized and incubated with 4 minimum haemolytic doses of glycerinated haemolytic horse serum for 30 min. before use.

The appropriate complement dilutions in 1 ml. volumes were put into sets of five tubes. To these were added 0·4 ml. of diluted serum and 0·2 ml. of antigen suspension. Control titrations of antigen and serum alone were included in the tests and the total volume of each tube was made up, where necessary, to 1·6 ml. with veronal buffer.

After incubation for 30 min. in a water bath at 37° C., 1 ml. of the haemolytic indicator system was added to each tube and incubation was continued for another 30 min. The red cells which had not been haemolysed in the tubes were then sedimented by centrifugation and the degree of haemolysis in each tube determined with a simple colorimeter.

Titration of antisera

Each serum was titrated within a 1·25-fold dilution series from 1/32 to 1/298 against a 1/10 suspension of each antigen. The volume of complement required for 50 % haemolysis after absorption by the antigen–antibody complex was determined graphically by plotting the percentage haemolysis between 20 and 80 %

against the log. dose of complement in the tubes. The log. dose of complement required for 50 % haemolysis was indicated by a line drawn between two adjacent points above and below 50 %. Control titrations of the antigen, serum and complement were made in each test.

Assay of complement-fixing activity

The complement-fixing activity of the antisera with the antigens was calculated by the method described by Bradish *et al.* (1960) using the formula

$$\alpha B = (V_i - V_0)/v_i.$$

V_i is the volume of complement required for 50 % haemolysis in the test, V_0 is that volume of complement required for 50 % haemolysis by the most anticomplementary reagent, usually the antigen, in the control tests and v_i is the volume of whole serum used in the test. The complement-fixing activity αB is thus expressed as the volume of complement fixed per unit volume of serum.

When the complement-fixing activity of an antiserum at all the points in a dilution series is calculated in this way, it is found that there is depression of fixation when antibody on the one hand and antigen on the other is in excess in the reacting mixtures. There are, however, two or three points within the dilution series which exhibit close proportional relationship to one another. The amounts of complement fixed at these points can be used to calculate a mean value for the reaction slope which expresses the specific reaction of the antibody with the antigen in the test when these are both present in optimum proportions for maximum fixation of complement.

The reaction slope can be plotted as in Fig. 1 by using the equation above and choosing an appropriate value for v_i. Thus,

when $$\alpha B = (V_i - V_0)/v_i,$$

then $$V_i = (\alpha B \times v_i) + V_0.$$

Cross-fixation ratios

In practice, a number of strain-specific antisera are titrated with one preparation of antigen and the complement-fixing activity of each serum is calculated by the method described. When a strain-homologous antiserum prepared from the antigen is included in the range of sera tested, the complement-fixing activity of each antiserum can be expressed as the cross-fixation ratio of each serum to that of the homologous strain-specific serum.

Thus, when two antisera, A (homologous) and B (heterologous), react with one preparation of antigen, the cross-fixation ratio of heterologous serum B to homologous serum A equals

$$\frac{\text{complement-fixing activity of serum } B}{\text{complement-fixing activity of serum } A}.$$

The values obtained are found to be highly specific to each serum and this specificity is not affected by variations in the concentration of antigen or the components of the haemolytic system.

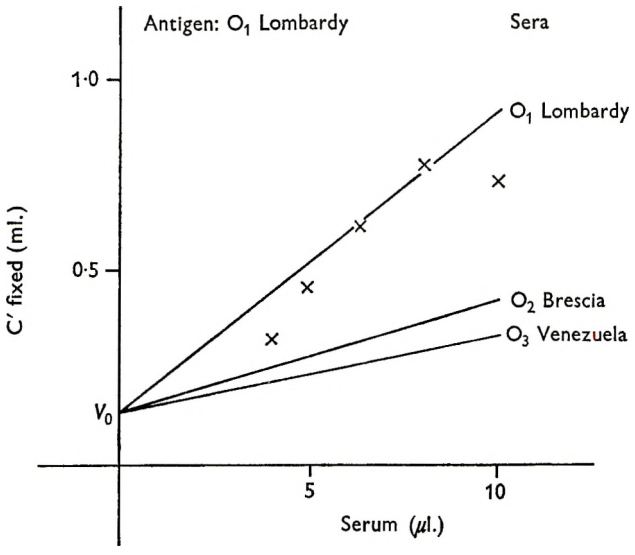

Fig. 1. The titration of three subtype specific sera in the presence of one antigen.

Cross-fixation products

When a series of strain-specific sera is tested with the virus antigens used to produce the sera, and the amounts of complement fixed by homologous and heterologous mixtures of antigen and antibody reacting in optimum proportion are compared as cross-fixation ratios, the relationship of the antigens to each other can be expressed as the product of the cross-fixation ratios of their strain-specific sera. Identical strains of virus have cross-fixation products of 1·0 while antigenically different strains have cross-fixation products of less than 1·0, depending upon the degree of difference.

RESULTS

Serum titrations

Table 1 shows the results of a complement fixation test in which a serum prepared from O_1 Lombardy virus is titrated in the presence of its homologous antigen. The volumes of complement fixed at the second and third dilutions of the series are proportionately greater per unit volume of serum. The inhibition of fixation in the first reaction is caused by antibody excess and that in the last two reactions by antigen excess. The second and third readings are therefore used to calculate the mean complement fixation by the serum reacting with this antigen when the two are present in optimum proportions.

When a range of strain-specific sera had been prepared, a series of complement fixation tests was set up in which all the sera were titrated in parallel with each of the antigens used to prepare the sera. Figure 1 illustrates a test in which three sera were titrated with O_1 Lombardy antigen.

Cross-fixation ratios

In each test the homologous serum–antigen reaction was included and the amounts of complement fixed by the heterologous reactions were expressed as cross-fixation ratios of the homologous reaction. The expression of the results of the tests

Table 1. *The assay of the complement-fixing activity of O_1 Lombardy antiserum with O_1 Lombardy virus (Fig. 1)*

	Reacting mixtures. Constant antigen + serum dilutions	μl. serum in test (v_i)	ml. C′ fixed in test (V_i)	Actual ml. C′ fixed ($V_i - V_0$)	ml. C′ fixed/ml. serum $\frac{V_i - V_0}{v_i}$	
1	1/40	10·0	0·741	0·623	0·062	
2	1/50	8·0	0·776	0·658	0·082*	
3	1/62·5	6·4	0·624	0·506	0·079*	
4	1/78	5·1	0·442	0·324	0·064	
5	1/98	4·1	0·320	0·202	0·049	
6	Antigen control (no serum)	0·0	0·118	—	—	V_0
7	Serum control 1/40 (no antigen)	10·0	0·084	—	—	
8	C′ control (no antigen, no serum)	0·0	0·080	—	—	
				Sum 2 and 3	0·161	
				Mean 2 and 3	0·081	

$\therefore$ Complement-fixing activity, αB, of O_1 Lombardy serum with O_1 Lombardy virus = 0·081 ml. C′/μl. antiserum,

and V_i at serum dilution 1/40 (10μl.) $= (\alpha B \times v_i) + V_0$,

$= (0{\cdot}081 \times 10) + 0{\cdot}118.$

$= 0{\cdot}928$ ml. C′ dilution.

(1) v_i is the volume of undiluted serum used in the test, V_i is the volume of complement required for 50 % haemolysis, and V_0 is the volume of complement for 50 % haemolysis in the most anticomplementary control, the antigen.

(2) Reaction No. 1 exhibits antibody-excess inhibition of complement fixation and Nos. 4 and 5 antigen-excess inhibition. These results are not, therefore, included in the calculation of complement-fixing activity at optimum proportions of antiserum with antigen.

* Region of maximal proportional fixation.

as ratios has the advantage that repeated tests with different preparations of the same antigen and different preparations of the haemolytic system are standardized as a proportion of the homologous reaction in each test. Table 2 gives the cross-fixation ratios of six antigens reacting in a series of tests with the six antisera prepared from these antigens.

Cross-fixation products

When the cross-fixation ratios of the six sera reacting with the six antigens had been determined, the antigenic relationship between pairs of antigens was expressed as the product of their cross-fixation ratios, one with the other (Table 3). Antigens O_1, O_2, O_3 and O_6 have cross-fixation products of less than 0·5 with one another and are therefore widely different. Antigen number 1411, however, has a cross-fixation product of 0·96 with antigen O_2 and the two virus strains have a close antigenic relationship. This relationship is reflected in the cross-fixation products of this antigen with the other virus strains. Antigen number 1698 is more difficult to classify as it exhibits some relationship with the two strains O_1 and O_2.

The use of the cross-fixation product to assess the antigenic relationship between strains of virus is of value when using strain-specific sera which may have a wide

Table 2. *The cross-fixation ratios of six strain-specific antisera reacting with the six virus strains used to produce the sera*

Virus antigens	Antisera					
	O_1	O_2	O_3	O_6	1411	1698
O_1	1·0	0·37	0·265	0·9	0·57	1·4
O_2	0·83	1·0	0·24	1·23	1·85	1·49
O_3	0·76	0·24	1·0	1·04	0·24	0·96
O_6	0·37	0·08	0·2	1·0	0·2	0·47
1411	0·47	0·52	0·15	0·81	1·0	0·86
1698	0·44	0·36	0·16	0·54	0·59	1·0

Table 3. *Cross-fixation products showing the antigenic relationship between virus strains*

O_1	1·0					
O_2	0·31	1·0				
O_3	0·2	0·06	1·0			
O_6	0·33	0·10	0·21	1·0		
1411	0·27	0·96	0·04	0·16	1·0	
1698	0·61	0·54	0·15	0·25	0·51	1·0
	O_1	O_2	O_3	O_6	1411	1698

range of homologous antibody titre. Virus strain O_6, which is used to produce stock type-specific serum, has had many passages in guinea-pigs and does therefore produce a serum of very high antibody titre. This is reflected in the high levels of fixation produced with heterologous antigens of the same type. When, however, O_6 antigen reacts with the heterologous sera the levels of fixation are low and the antigenic variation between the strains of virus becomes apparent.

Over twenty strains of virus of type O have been examined by this technique. Antigenic differences such as those exhibited by subtype strains O_1, O_2 and O_3 have been demonstrated in several of the strains examined and indicate the existence of at least eleven subtype groups within immunological type O. There were several strains such as BFS 1411 which could be classified as identical or very closely related to the subtype reference strains but there were other strains such as BFS 1698 which could not be classified with such certainty. A classified list of virus strains of Type O is given in Table 4. These virus strains have been recovered from epizootics in the field and some have been used for vaccine production in different parts of the world. The strains were examined at the World Reference Laboratory for Foot-and-Mouth Disease at Pirbright, using the complement-fixation technique described.

DISCUSSION

These experiments describe the development of a technique for the definition of subtype differences between strains of virus of one immunological type. The complement fixation test has been found preferable to other laboratory techniques such as mouse serum neutralization tests, agar gel diffusion or the colour test, which do not provide reproducible results of the same precision. The best results were

Table 4. *The classification of strains of foot-and-mouth disease virus of Type O*

Subtype no.	Designation of strain	Origin of material examined by W.R.L.	Other references to subtype differentiation
O_1	Lombardy	Cattle epithelium ex Tübingen, Germany,* 1958	Traub & Möhlmann (1946); Schneider (1950)
	M11	Field sample ex Mexico, 1952	Graves (1960)
	BFS 1698	British Field Sample, 1961	—
O_2	Brescia	Cattle epithelium ex Tübingen, Germany*	Ubertini (1951, 1954); Schneider (1950); Girard, Mackowiak & Robin (1952)
	Italian O '1950'	Cattle epithelium ex Brescia, Italy†	—
	Channel Islands 1/57	W.R.L. sample	Graves (1960)
	BFS 1312 BFS 1411 BFS 1426 BFS 1428 BFS 1429	British Field Samples: 5 in 1958, 1 in 1959, 1 in 1960	—
O_3	Venezuela	Cattle epithelium ex Tübingen, Germany*	Schneider & Kosch (1951); Michelsen & Thiesen (1951)
	Ven 1	Field Sample ex Venezuela, 1950	Graves (1960)
O_5	India	Mukteswar‡ Goat 'O', 1961	—
	India 1/62	W.R.L. sample	—
O_6	Pirbright	O_{v_1} British Field Sample, 1924. Reference guinea-pig strain used for production of type-specific antiserum for use by W.R.L.	—
O_7	Italy 1/58	Cattle epithelium ex Brescia, Italy†	Nobili (1962)
	Poland 1/59	W.R.L. sample	—
O_8	Brazil 1/60	Cattle epithelium ex Bahia, Brazil,§ 1960	New subtype reported by Pan-American Foot-and-Mouth Disease Centre and confirmed by W.R.L.
O_9	Kenya 102/60	W.R.L. sample	—
O_{10}	Philippines 3/58	W.R.L. sample	—
O_{11}	Indonesia 1/62	W.R.L. sample	—

Samples sent for identification to the World Reference Laboratory for Foot-and-Mouth Disease (W.R.L.) are given a reference number which indicates the country of origin, the serial number of the sample and the year of collection.

Reference strains supplied by other Institutes

* Bundesforschungsanstalt für Viruskrankheiten der Tiere, Waldhäuser Höhe, Tübingen, Germany.

† Istituto Zooprofilattico Sperimentale delle Provincie Lombarde, Brescia, Italy.

‡ Indian Veterinary Research Institute, Mukteswar-Kumaon, Uttar Pradesh, India.

§ Pan American Foot-and-Mouth Disease Centre, Rio de Janeiro, Brazil.

obtained with fresh antigen of high infectivity and antiserum of high antibody titre. While the antibody level of serum was found to be stable under normal conditions of storage, antigens prepared on different occasions produced different levels of fixation. Minor variations in the concentration of antigen or in the reagents used in the haemolytic system would not, however, upset the specificity of the reactions if the complement-fixing activity of the reacting mixtures of antigen and antibody were expressed as cross-fixation ratios.

If the antigenic relationship of two virus strains has to be determined, it is necessary to prepare strain-homologous antisera and to compare the cross-fixation ratios of both sera tested with both antigens. A study of the results tabulated in Table 3 demonstrates that the complement-fixing activity of an antiserum is a reflexion of two properties, its specificity in relation to the test antigen and also its antibody titre. Thus O_6 antiserum, because of its high antibody titre, exhibits a high cross-fixation ratio with all the virus antigens. The other sera, on the other hand, have low cross-fixation ratios with O_6 antigen and the subtype differences, expressed as cross-fixation products, become evident. O_6 serum is therefore very useful in a routine typing test because it gives a high level of fixation with a wide range of Type O antigens, but it would be unwise to assume that strong fixation with this serum would necessarily indicate close antigenic relationship.

When a collection of subtype virus strains and strain-specific reference sera has been prepared and their homologous and heterologous reactions one with the other are known, an unknown virus strain can be classified fairly rapidly. A strain-specific antiserum is prepared from the unknown virus in 6–8 weeks and the virus is then tested with its homologous and the known reference subtype sera. A comparison of the results with those of the stock subtype strains will show whether the virus under investigation is likely to be placed within a known subtype group or not. If it can be so placed, the stock virus will react with the new serum as if it were homologous and the cross-fixation product will not be significantly different from unity.

Virus strains have therefore been classified into subtype groups in which the cross-fixation product of each strain within the group is 0·5 or more with the reference subtype strain. While this level of cross-fixation product has been arbitrarily fixed, it is considered that a vaccine produced from one strain within the group will give good protection against challenge by a field strain of virus of the same group classification. This technique for the classification of virus strains has been applied to strains of other types by Davie (1962) and has confirmed the results of other workers who, using a variety of techniques, have reported subtype differences.

SUMMARY

1. A complement fixation test has been developed to measure the minor antigenic differences which occur between strains of the virus of foot-and-mouth disease of the same immunological type.

2. Strain-specific sera were prepared from each of the virus strains examined and then titrated with each of the antigens.

3. The amounts of complement fixed in each reaction were measured in the region of maximal fixation where the antigen and antibody were present in optimal proportions.

4. Heterologous antigen–antibody reactions were compared with the homologous reaction in each test and expressed as cross-fixation ratios.

5. Pairs of antigens could then be compared by taking the product of their cross-fixation ratios one with the other. The values of cross-fixation product so obtained ranged from 1·0 between identical strains to 0·01 between strains of different immunological type.

6. Strains of virus which exhibit cross-fixation products of 0·5 or more with one another have been classified within subtype groups.

I wish to acknowledge the guidance and encouragement of Dr J. B. Brooksby, Director of the Animal Virus Research Institute, Pirbright, and the technical assistance of Mr E. Scoates.

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J. Hyg., Camb. (1964), **62**, 413
Printed in Great Britain

Cell-free varicella–zoster virus in tissue culture

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(*Received* 29 *April* 1964)

The cultivation of varicella–zoster (V-Z) virus in human tissue cultures was first reported by Weller & Stoddard (1952) and by Weller (1953). Infective virus was not detected in the fluid phase of cultures and serial propagation of virus could only be achieved by transfer of infected cells (Weller & Witton, 1953; Weller, Witton & Bell, 1958). This relationship between virus and tissue-culture cells was subsequently observed by several workers (see Downie, 1959). Weller (1958) also failed to obtain virus from infected cells by various disruptive procedures. However, Gold & Robbins (1958) reported briefly that they had produced a cytopathic effect in serial cultures of human amnion or monkey kidney cells by the use of cell-free tissue-culture material. Taylor-Robinson (1959) found that much of the infective virus in zoster vesicle fluids was cell-free in marked contrast to the absence of virus in varicella and zoster tissue-culture fluids. He mentioned, however, that free infective virus had, on occasions, been obtained by ultrasonic treatment of infected amnion cells. Furthermore, the presence of convalescent varicella or zoster serum in the fluid phase of infected tissue cultures inhibited the spread of focal lesions, indicating that infective virus was not entirely intracellular. Caunt (1963) has recently described experiments in which infective virus was liberated from infected human thyroid tissue cultures.

The first section of this paper describes this work in more detail. In thyroid cultures higher titres are obtained than in human amnion, virus can be detected in cell-free fluid and considerable amounts can be liberated from the cultured cells by sonic disintegration.

Our own and other workers' failure to find cell-free virus in earlier experiments suggested that virus released from cells in culture might have been inactivated at 37° C. before adsorption to uninfected cells. In Section II experiments to test this possibility are recorded and the possible role of interferon has been investigated.

MATERIALS AND METHODS

Viruses

Vesicle fluids from varicella and zoster patients were collected in tissue-culture maintenance medium and either used immediately or stored at −70° C. Inoculation of tissue cultures and serial propagation by passage of infected cells was carried out as described previously (Taylor-Robinson, 1959).

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Sera

The immune sera used in complement fixation and neutralization tests were convalescent zoster sera taken 2–3 weeks after the onset of the rash. A standard serum for complement fixation tests has been used in this laboratory for several years and is known to give high titres with potent vesicle-fluid or tissue-culture antigens.

Tissue cultures

Primary trypsin-dispersed cultures of human amnion cells were prepared by the method of Zitcer, Fogh & Dunnebacke (1955). About 5×10^5 cells per ml. were distributed in 1·0 ml. amounts to 6 in. $\times \frac{5}{8}$ in. tubes and in 20 and 35 ml. quantities to 6 and 12 oz. flat-sided medicine bottles respectively. Primary trypsin-dispersed cultures of human thyroid cells were prepared as described by Pulvertaft, Davies, Weiss & Wilkinson (1959) and were grown in similar containers.

Media

Amnion cultures were grown either in 10% inactivated horse serum and 10% tryptic digest broth in Hanks's saline with 0·03% bicarbonate or in 10% horse or lamb serum in Eagle's medium. Cultures were maintained in similar media but with the concentration of serum (horse, lamb or calf) reduced to 2 or 5%.

Thyroid cultures were grown in 5% calf serum in 199 medium with 0·06% bicarbonate and maintained in the same medium with 2% serum. All media contained 100 units/ml. of penicillin and 100μg./ml. of streptomycin.

Complement fixation tests

Complement fixation tests were performed as described by Taylor-Robinson & Downie (1959).

Ultrasonic disintegration of cells

Bottle cultures of amnion cells with extensive cytopathic changes were trypsinized with 0·25% 'Difco' trypsin in phosphate-buffered saline, centrifuged at 1000 r.p.m. for 5 min. and the cell deposits resuspended in growth medium. The cells from a 6 oz. bottle were usually resuspended in about 1 ml. of medium. These cells, in a container surrounded by crushed ice, were disrupted with an M.S.E. Mullard ultrasonic disintegrator. The vibrator probe of stainless steel had end diameters of $\frac{3}{4}$ in. and $\frac{3}{8}$ in. and a velocity increase ratio of 4:1.

Infected thyroid cells were usually removed from the glass with 0·02% versene in phosphate-buffered saline but sometimes 0·25% 'Difco' trypsin was used as well. Subsequent treatment was the same as that for amnion cells.

Filtration

In some instances tests for the presence of cell-free infectious virus were made by filtration of material after ultrasonic disintegration of cells. Filtration was through 'Oxoid' membranes with an average pore diameter of 0·5–1·0μ. The integrity of

the membranes was checked after each experiment by the filtration of *Chromobacterium prodigiosum* or *Escherichia coli* cultures; these filtrates were invariably bacteriologically sterile.

Ultracentrifugation

In some cases virus preparations were concentrated by centrifugation in a Spinco Model L centrifuge, Rotor No. 40 at 12,500 r.p.m. (14,000 g) for 30 min.

RESULTS

Section I

Comparison of human amnion and thyroid cells for the cultivation of V-Z virus

Human thyroid tissue cultures have been found to be more sensitive for the isolation of V-Z virus from vesicle fluids and to give higher titres in the titration of infected cell suspensions than human amnion cultures (see Table 1).

Table 1. *Comparison of the sensitivity of human amnion and human thyroid tissue cultures for the isolation and titration of V-Z virus*

Virus preparation	Number of focal lesions developing per culture tube of: Amnion	Thyroid
Vesicle fluid virus		
McG Varicella	0, 0	8, 3
Ho Zoster	31	63
Be Zoster	0, 0	40, 33
Mi Zoster	0, 0, 2, 0	6, 9, 9, 5
Infected cell suspension		
Mo Varicella 7th pass in thyroid	201, 210	400
Mo Varicella 9th pass in amnion	100, 68	323, 272

Human thyroid cultures infected with V-Z virus also appear to be a better source of soluble complement-fixing antigen than similar cultures in human amnion. The tissue-culture fluids from the latter require 3- to 10-fold concentration before they are useful antigens (Caunt, Rondle & Downie, 1961) but the unconcentrated fluids from infected thyroid cultures can be prepared and used in the following way. Cultures are inoculated heavily with V-Z virus (i.e. at least 500 infective units in a 6 oz. bottle) and, after 3–4 days when cytopathic changes are obvious, the normal maintenance medium is replaced by medium 199 with 0·09 % sodium bicarbonate and no serum. This medium is harvested after a further 5–7 days and may be replaced and a second harvest obtained if the cells are still viable. These fluids can then be used unconcentrated, or in some cases diluted up to 1/4, as antigen in complement fixation reactions (Table 2).

The absence of virus from the fluid phase of amnion tissue cultures

This phenomenon has been confirmed many times during the course of routine subculture. Occasionally the medium from bottle cultures of amnion cells showing

very extensive cytopathic changes has been centrifuged at 3000 r.p.m. for 10 min. and the top portion of the supernatant inoculated into amnion or thyroid cultures. Although a few focal lesions have sometimes occurred in the inoculated cultures, it is difficult to exclude the possibility that these are the result of virus within intact cells, or cell fragments, which did not sediment. If virus is free then it is present in amounts too small to be demonstrable in filtrates.

Table 2. *Complement fixation with antigens prepared in human amnion and human thyroid cells*

Antigen	Concentration or dilution	Dilution of zoster convalescent serum 1/16	1/32	1/64	1/128	Control (no serum)
203* (amnion)	× 10 concentration	+ + + +	+ + + +	+ + + +	+ + + +	−
	× 5 concentration	+ + + +	+ + + +	+ + +	+ +	−
225† (thyroid)	Unconcentrated	+ + + +	+ + + +	+ + + +	+ + + +	−
	½ dilution	+ + + +	+ + + +	+ + + +	+ + + +	−
	¼ dilution	+ + + +	+ + + +	+ + +	+ +	−

* Antigen 203 from Mi Zoster 7th passage in amnion cells maintained in 50% bovine amniotic fluid in Hanks's saline.

† Antigen 225 from Be Zoster 8th passage in thyroid cells maintained in medium 199.

+ + + +, complete fixation; + + +, + +, 25, 50% lysis; −, complete lysis.

The repeated subculture of V-Z virus in amnion cell cultures has not resulted in the appearance of virus in the fluid phase. Several strains of virus were subcultured for 10 or more passages and the varicella strain C.D.T. which was previously reported (Taylor-Robinson, 1959) to have undergone 33 subcultures has now been through 109 subcultures without infective virus being detected in the tissue-culture medium.

Presence of free virus in the fluid phase of thyroid tissue cultures

In thyroid tissue cultures 10–14 days after inoculation with V-Z virus, very small, presumably secondary, focal lesions occur in addition to the primary foci, which are very much greater in diameter by this time. This suggested the presence of infective virus in the culture fluids and with several virus strains this has now been demonstrated. The strains were derived from both varicella and zoster cases and with a heavy inoculum free virus was first demonstrated after 3 days, but continued to be released until the cells disintegrated. The titre of virus in the fluid was not high but the virus concentration could be increased by ultracentrifugation. This virus was filterable and was neutralized by immune serum (Table 3) in the same way as virus from vesicle fluid.

Effect of ultrasonic disintegration of infected tissue cultures

Numerous experiments were carried out in an endeavour to release virus from infected amnion and thyroid cells. Preliminary experiments showed that ultrasonic treatment of vesicle fluid halved the virus content for each minute up to 2 min. (Table 4). We assumed that, if similar virus was liberated from tissue-

Table 3. *V-Z virus in thyroid tissue-culture fluids*

	Number of focal lesions developing from 1 ml. of	Virus strain McC 23	Virus strain McC 24
A	Supernatant fluid after centrifuging 1000 r.p.m. 5 min.	46, 42	6, 4, 5
B	Fluid from *A* after filtration	2, 6	2, 1, 1
C	Fluid from *A* concentrated × 70 by ultracentrifugation	NT	100, 110
D	Fluid from *C* + 0·1 ml. 1/5 immune serum	NT	0, 0
E	Fluid from *B* + 0·1 ml. 1/5 immune serum	0, 0	NT

NT, Not tested.

Table 4. *Effect of ultrasonic treatment on virus in zoster vesicle fluids*

Zoster vesicle fluids	Number of focal lesions produced in amnion cultures by vesicle fluids after ultrasonic treatment for 0 sec.	15 sec.	1 min.	2 min.	5 min.	10 min.
ZHA	141	116	NT	NT	NT	NT
ZHO	31	NT	17	8	3	2

culture cells, half of it also would survive 1 min. of treatment and some would survive 2 min. When infected amnion cells were subjected to this treatment for various periods up to 2 min., titration of the cell debris showed that there was a marked reduction in infectivity compared with the original infected cell suspension (Table 5). However, on several occasions filtrates of the disrupted cell suspension contained small quantities of infective virus. The amount of virus obtained by this procedure was too small to be of practical value as a source of cell-free virus in the laboratory.

Table 5. *Effect of ultrasonic disintegration of infected human amnion cells*

Virus strain and passage	Number of focal lesions produced in amnion cultures per ml. of: Original infected cell suspension	Suspension of cell debris after treatment for 15 sec.	30 sec.	45 sec.	1 min.	1½ min.	2 min.
Th (Varicella) 62	500	13	1	0	NT	NT	NT
	54	0	NT	(2)†	NT	NT	NT
	500	NT	17 (1)†	NT	NT	NT	NT
Mo (Varicella) 9	3000	50	25	15	20	40	NT
Wr (Varicella) 4*	580	NT	NT	NT	NT	NT	55 (5)†

* In this experiment the titration was in thyroid cultures.
† Figures in brackets = number of focal lesions per ml. of material after filtration.
NT, Not tested.

When thyroid cultures were similarly treated there was little loss of infectivity. If the cell debris suspension was centrifuged at 3000 r.p.m. for 5–10 min. some of the infectivity was lost with the sediment but filtration of the supernatant had little effect on the virus titre (Table 6, first line). Supernatant fluids with titres of

10^4–10^5 infectious particles per ml. have been obtained and this has proved to be a practical method of obtaining virus for use in neutralization tests. Such preparations are also excellent complement-fixing antigens and one such as that from J 9 in Table 6 when used diluted 1/10 gave complete fixation with 1/256 dilution of the convalescent zoster serum used in Table 2. This cell-free virus has also been stored at −63° C., when some loss of infectivity occurs. It is not yet known whether this loss is related to the length of storage but in the case of vesicle-fluid virus a similar loss occurs; the extent of this loss varies but has no apparent relationship to length of storage.

Table 6. *Effect of ultrasonic disintegration of infected human thyroid cells*

	Titre expressed as number of focal lesions produced in thyroid culture per ml.					
					Materials after storage at −63° C.	
Virus strain and passage	Original infected cell suspension	After 2 min. ultrasonic treatment	Supernatant after centrifuging treated material	Filtrate of treated material	Titre	Days of storage
McC (Zoster) 8	2×10^5	9×10^4	2×10^4	2×10^4	$1{\cdot}3 \times 10^3$*	8
Mo (Varicella) 5	4×10^3	NT	NT	2×10^3	1×10^2*	14
Mo (Varicella) 7	$2{\cdot}4 \times 10^4$	3×10^4	NT	5×10^3	NT	—
J (Varicella) 9	2×10^5	NT	$1{\cdot}6 \times 10^5$	NT	6×10^4†	10
					$2{\cdot}6 \times 10^4$†	84

* Filtrates.
† Unfiltered supernatant fluids.

Section II

In considering the low titre or complete lack of free virus in the fluid phase of tissue cultures, it seemed possible that under normal tissue-culture conditions small amounts of virus might be released but be inactivated at 37° C. before they can be readsorbed to normal cells. The following experiments were carried out to test this possibility.

Rate of inactivation of virus at 37° C.

Virus was diluted in tissue-culture maintenance medium and seven 2 ml. samples of this material were incubated at 37° C. At various times up to 15 hr. successive samples were inoculated in 1 ml. amounts into thyroid cultures. The results in Table 7 show that there was no appreciable inactivation of vesicle-fluid virus within 9 hr. and little within 15 hr., although with virus grown in tissue culture only about 60 % survived for 9 hr.

Rate of adsorption of virus to tissue-culture cells

The rate of adsorption of virus to amnion and thyroid cells in tissue culture was tested using either varicella or zoster vesicle fluid or virus obtained by the disintegration of infected thyroid cells. In one of these experiments 0·25 ml. of zoster vesicle fluid was diluted to 7 ml. with tissue-culture maintenance medium and inoculated in 0·1 ml. quantities into 7 tube cultures of amnion cells; these cultures

Table 7. *Rate of inactivation of virus at* 37° C.

Virus	Time (hr.)	Number of focal lesions/ml. of virus suspension	Average number/ml.	Survival (% of count at 0 hr.)
Varicella vesicle fluid	0	75, 81	78	100
	3	97, 78	87	—
	6	62, 87	74	94·9
	9	73, 71	72	92·3
	12	63, 52	57	73·1
	15	46, 51	48	61·5
Varicella 9th pass from thyroid cells	0	108, 111	109	100
	3	109, 81	95	87·1
	6	55, 54	55	50·5
	9	67, 59	63	57·8

are referred to as 'original tubes'. Immediately after inoculation the fluid was removed from one of the original tubes and placed in a fresh tube (*A*). Then the original tube (*O*) was rinsed with 1 ml. of fresh medium which was rocked 30 times before it was removed to a fresh culture (*B*); 1·0 ml. of fresh medium was then added to the original tube. These three tubes, and the remaining 6 original tubes, were then incubated at 37° C. This procedure of removing the medium and rinsing out the tube was carried out on successive cultures at 3-hourly intervals up to 15 hr. and the seventh culture was left as a control. The results recorded after 5 days' incubation at 37° C. are shown in Table 8 (*a*). The amount of virus adsorbed (tube *O*) and the amount removed from the cultures at each period of time was known (tubes *A* and *B*), so that the percentage of virus fixed at each time interval could be calculated. The results of a similar experiment using varicella vesicle fluid and thyroid cells are shown in Table 8 (*b*), and of using virus from thyroid cultures and thyroid cells in Table 8 (*c*). It can be seen that in these experiments 93, 85 and 84 % respectively of the virus inocula were adsorbed in 9 hr. and the virus which was not adsorbed remained infective for fresh cultures. In experiment (*b*) of Table 8 a duplicate set of original tubes was set up and a zoster convalescent serum was added at the various time intervals to neutralize free virus. In these tubes the maximum number of infected foci was attained by 9 hr., thus confirming that the virus inoculum was adsorbed within that time. It is noticeable, however, that the number of infected foci developing under immune serum was less than those in tubes without immune serum. This suggests that, although the virus was adsorbed to the cells within 9 hr. and could not then be washed off, some of it remained at the surface of the cells and could be neutralized by immune serum even after 12 or 15 hr.

These results, together with the observations on inactivation of the virus at 37° C., show that most of the virus inoculum is adsorbed to the cells within 9 hr. and that little inactivation occurs within this period. This suggests that if virus is liberated from infected cells in tissue culture it is unlikely to be inactivated at the temperature of incubation before being adsorbed to normal healthy cells.

Table 8. *The rate of adsorption of virus to tissue-culture cells*

Virus	Tissue culture	Time (hr.)	Number of focal lesions/tube			Total number of focal lesions	% virus adsorbed	Duplicate tube + immune serum
			O	*A*	*B*	(*T*)	(*O*/*T* × 100)	
(*a*) Zoster vesicle fluid	Amnion	0	6	311	11	328	2	—
		3	190	140	21	351	54	—
		6	155	86	20	261	60	—
		9	430	14	17	461	93	—
		12	412	5	2	419	98	—
		15	410	1	2	413	99	—
		Virus control	415	—	—	415	—	—
(*b*) Varicella vesicle fluid	Thyroid	0	2	35	5	42	5	0
		3	38	40	4	82	46	22
		6	55	10	1	66	83	33
		9	58	10	0	68	85	46
		12	59	2	2	63	94	36
		15	73	1	1	75	97	42
		Virus control	78	—	—	78	—	—
(c) Varicella 9th pass from thyroid cells	Thyroid	0	4	128	8	140	2·9	—
		3	87	86	22	195	44·6	—
		6	137	30	10	177	69·5	—
		9	111	27	4	142	84·1	—
		Virus control	111	—	—	111	—	—

O, Original tube, incubated for time shown; *A*, medium removed from original tube after time shown, to a fresh culture; *B*, rinsings of original tube, removed to a fresh culture.

Attempts to demonstrate interferon production

A possible explanation of the inability to detect free infective virus in the fluid phase of infected amnion cultures, and for the absence of secondary foci, was that the infected cells produced interferon. We attempted to demonstrate the presence of interferon in amnion and thyroid cultures heavily infected with V-Z virus, i.e. cultures in which 75 % or more of the cell sheet showed cytopathic changes. The medium from such cultures was centrifuged at 1000 r.p.m. for 5 min. and the supernatant was added to cultures of amnion or thyroid which were then incubated for 24 hr. at 37° C. Virus from vesicle fluid, or in one case from thyroid tissue cultures, was then added to some of the 'interferon-treated' cultures and to control cultures not so treated. The results of these experiments are shown in Table 9. There is no evidence of interferon production in any of the tests. The fluid from infected cultures was also tested for interferon in chick embryo fibroblast tissue cultures using Sindbis as indicator virus and no interferon was demonstrated. It therefore appears that if interferon is produced in infected cultures there is not enough to account for the absence of free virus or of secondary focus formation.

Table 9. *Attempts to demonstrate interferon production in infected tissue cultures*

Methods used to produce interferon				Test for presence of interferon				
						Number of focal lesions per culture		
Tissue cultures	Inoculum for cultures	Period of incubation (days)	Time of contact of medium used as 'interferon' (days)	Virus	Tissue culture	Untreated culture + virus	'Interferon'-treated culture + virus	'Interferon'-treated culture (no virus)
Amnion	Zoster 11th pass	13	7–13	Zoster V-F	Thyroid	32	29	0, 0
Thyroid	Varicella 1st pass	20	13–20	Zoster V-F	Thyroid	26	55	0, 0
Amnion	Varicella 11th pass	7	4–7	Varicella V-F	Amnion	72	147, 190	9, 4
Amnion	Varicella 11th pass	7	4–7	Varicella V-F	Amnion	72	69	1, 3
Amnion	Varicella 11th pass	7	4–7	Varicella* 8th pass	Amnion	142	105, 85, 85	1, 3

* Virus prepared by ultrasonic disruption of infected thyroid cells.
V-F, Vesicle fluid.

DISCUSSION

The most striking aspect of our experiments has been the ability to obtain cell-free, infective virus in small quantities in the fluid phase of thyroid tissue cultures and in much larger amounts by the disintegration of such cultures. This is in marked contrast to our experience with amnion cells and that of previous investigators using amnion, human embryo fibroblasts and HeLa cells (Weller & Witton, 1953; Weller *et al.* 1958; Taylor-Robinson, 1959; Rapp & Benyesh-Melnick, 1963). Little or no free infective virus occurs in the fluid phase of infected amnion cultures and although small amounts can be released by ultrasonic disintegration of these cells much greater quantities (at least 100 times as much) are obtained by a similar treatment of thyroid cells.

Weller *et al.* (1958) suggested that the virus within tissue-culture cells exists in an 'immature' non-infective form and that death of the cell results in loss of this virus. In both infected amnion and thyroid cells there is some loss of infectivity on disintegration of the cells but this loss is very much less in the case of thyroid cells. Even allowing for the fact that there may be greater viral synthesis in thyroid cells, a view which is supported by the greater production of soluble complement-fixing antigen by these cells than by amnion cells, it seems likely that the difference in the yield of free virus by the two cell systems is not a purely quantitative one and that a greater proportion of the virus in infected thyroid cultures is present in a complete or mature form. Some of this mature virus is in fact released into the supernatant fluid.

Tournier, Cathala & Bernhard (1957) observed particles with the electron microscope, presumably virus particles, outside as well as within human embryonic fibroblasts infected with varicella. The infectivity of these particles was not determined but the observation does suggest that virus is released from these infected cells. We have investigated the possibility that small amounts of virus might be liberated from amnion cells and that this virus would be inactivated at 37° C. before it could be adsorbed to healthy cells. Our observations indicate that this is unlikely to be the case. We have also failed to demonstrate an interferon-like substance in sufficient amounts to protect healthy cells in tissue culture. This is not surprising since foci continue to increase in size over many days in monolayers of amnion and if interferon were formed it might be expected to protect cells adjacent to the infected ones.

It has previously been noted (Weller *et al.* 1958; Taylor-Robinson, 1959) that the presence of zoster or varicella immune serum in the medium of tissue cultures does not prevent the development of infected foci in monolayers inoculated with infected cells, although it does neutralize vesicle fluid virus and, as we have shown, cell-free virus from tissue cultures. The foci which develop from infected cells under antiserum, however, are smaller in size, suggesting that spread of infection by extracellular virus, which can be affected by antiserum, normally does occur.

It is interesting to compare these findings with those relating to the growth of herpes simplex in HeLa cells (Stoker, 1959). In this work single infected HeLa cells were found to be capable of initiating foci of infection in HeLa cell monolayers

even in the presence of antiserum, although they did not release infective virus if they were grown in isolation in microdrops. It was postulated that virus was present in the infected cells in a 'vegetative' state and that if released into the medium it lacked the power to penetrate new cells and therefore could not be detected. However, in monolayer cultures this vegetative virus was able to spread, particularly by giant cell formation in which there was fusion of contiguous cells. Formation of giant cells is also a feature of the growth of V-Z virus in monolayers of amnion and thyroid but at the periphery of infected foci there are always some infected cells which retain their individual integrity. The virus must therefore be able to infect intact cells and spread is not simply accomplished by the fusion of cells.

Recently Slotnick & Rosanoff (1963), using the fluorescent antibody technique of Weller & Coons (1954), have shown that varicella antigen is present in the cytoplasmic strands which join the cells and it is possible that infection of adjoining cells occurs via these strands before the infected cell or giant cell separates completely from its neighbours.

Other recent work which may be relevant concerns the isolation of infectious DNA. Such DNA from polyoma virus is capable of initiating plaque formation in monolayer tissue cultures (Weil, 1961), and DNA from papilloma virus can produce papillomas in the skin of rabbits and is unaffected by concentrations of antiserum which completely inactivate papilloma virus suspensions (Ito, 1960).

It might be suggested that V-Z virus exists inside amnion cells as infectious DNA rather than as complete virus and that this can infect adjacent cells, but if it is released from the cell it combines with non-viral protein in the tissue culture medium and hence becomes non-infective. This would explain the loss of infectivity on disruption of the cells but does not explain the partial inhibition of spread of virus under antiserum since the DNA would not be neutralizable by immune serum. Little is known of the changes which take place in cells infected with V-Z virus and it is possible that further cytological and electron-microscopic studies might be helpful in elucidating the means of cell-to-cell transfer of infection and also in showing what differences there are between virus formation in thyroid and in other susceptible cells.

SUMMARY

Infected human thyroid tissue cultures have been found to yield free V-Z virus on ultrasonic disintegration and some virus is also present in the fluid phase of intact tissue cultures, while the yield of virus from infected human amnion cells is very low and no free virus is found in the fluid phase. Thyroid cultures are more susceptible to infection with V-Z virus, and the infected cultures are a better source of complement-fixing antigen, than are amnion cultures.

The lack of free virus in infected amnion tissue cultures cannot be attributed to protection by interferon. It also seems likely that if any infectious virus were released it would be more likely to be adsorbed to normal cells than to be inactivated, but as no secondary foci appear in amnion monolayers we conclude that infective virus is not released into the medium.

It is suggested that the virus in thyroid cells is present in a more complete form than that in amnion cells.

We are grateful to Dr R. M. Rawcliffe of Birkenhead General Hospital and Dr F. Whitwell of Broadgreen Hospital, Liverpool, for arranging the supply of thyroid tissue.

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J. Hyg., Camb. (1964), **62**, 425
Printed in Great Britain

The isolation of an unclassified virus from an outbreak of infantile diarrhoea

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(*Received* 11 *May* 1964)

INTRODUCTION

The role of viruses in human infantile diarrhoea is still obscure. Planned studies by different workers (Ramos-Alvarez & Sabin, 1958; Sommerville, 1958; Joncas & Pavilanis, 1960; Walker *et al.* 1960; Yow, Melnick, Blattner & Rasmussen, 1963) have resulted in conflicting reports and in only two instances have the number of isolations of enteroviruses from the test group been significantly greater than those from the control group. Undoubtedly there are viruses which have escaped detection to date, but the rapid increase in the practice of virology and the gradual improvement in techniques make it certain that many 'new' viruses will be isolated in the next few years. Some of these may be of significance in infantile diarrhoea. Recent reports (Duncan, 1960; Cooney, McLaren & Bauer, 1962; Abrahams, 1963) already indicate the existence of several enteroviruses which cannot be classified as members of the existing subgroups (Polio, Echo and Coxsackie).

These unclassified viruses have a number of characteristics in common, the most notable of which is their inability to produce cytopathic changes in cultures of monkey kidney epithelium. They are non-pathogenic for unweaned mice but are readily cytopathic when inoculated into tissue cultures of human origin.

Investigations into an outbreak of diarrhoea in a Children's Home resulted in the isolation of a virus with characteristics of this latter group and which is serologically distinct from the recognized serotypes of the enterovirus groups.

MATERIALS AND METHODS

Tissue cultures

H.Ep II cells and HeLa cells were propagated in four ounce McCartney bottles in a medium consisting of 10% human serum, 5% calf serum, 0·5% lactalbumin hydrolysate and 84·5% Hanks's balanced salt solution. Monkey kidney cultures were obtained from Commonwealth Serum Laboratories, Melbourne, and were maintained in Medium 199 and 0·5% bovine albumin. Cultures of human amnion, human embryonic kidney, rabbit kidney and guinea-pig embryonic kidney were prepared by trypsinization. Chick and mouse embryo fibroblasts were grown from explants and human embryonic fibroblasts were propagated according to the method of Moorhead & Hayflick (1961). All cell cultures except monkey kidney were in a medium of 5% calf serum, 0·5% lactalbumin hydrolysate and 94·5% Hanks's balanced salt solution at the time of inoculation.

Sources of viruses and antisera

Antisera to poliovirus types 1–3, Coxsackie B virus types 1–6 and Echo virus types 1–25 were obtained from Microbiological Associates, Bethesda, and antisera to Adenovirus types 1–17 and 19–24 were obtained from Italdiagnostic Ltd. Antisera to Coxsackie A viruses 1–10, 20 (*a*), 20 (*b*) and 21, Echovirus types 26, 27, 28, F.E.B. and 1266 were prepared in this laboratory from prototype strains. Coxsackie A viruses 11–19, 22 and 24 were received from Dr Duxbury of the Commonwealth Serum Laboratories. Viruses Hu 39, Hu 659, Hu 2220 and C-Thai 18 with their homologous antisera were kindly sent by Dr A. Abrahams, Pittsburgh, U.S.A.

Neutralization tests

Specific antisera diluted to contain 50 antibody units per 0·1 ml. to homologous virus were mixed with an equal volume of virus suspension diluted to contain 100 ID 50 per 0·1 ml. and allowed to stand at room temperature for 2 hr. Tissue cultures were than inoculated with 0·2 ml. of this mixture. Virus was considered neutralized if no cytopathic effect (C.P.E.) was observed when 75 % or more of the cells in the virus control cultures showed degeneration.

Preparation of antiserum against prototype strain

One of the fifteen isolates was selected for the preparation of antiserum and determination of physical and biological properties. This virus was designated the 'Mill' strain. It was poorly antigenic in the rabbit and a prolonged course of inoculations was necessary to elicit an antibody response. Two rabbits were each given twelve intravenous inoculations of 5 ml. of tissue culture fluid at weekly intervals and also two intramuscular inoculations of 5 ml. Each rabbit thus received 70 ml. of tissue culture fluid. The rabbit serum had a titre of 64 against 100 ID 50 virus.

Preparation of tissue-culture fluid for estimation of particle size

Harvested tissue-culture fluid was clarified at low-speed centrifugation (2000 r.p.m. for 10 min.). Fluorocarbon (10 %, v/v) was added and the mixture homogenized in a Waring Blendor for 90 sec. Further clarification was carried out at 2500 r.p.m. for 15 min. The clear supernatant was used for filtration through Millipore membranes of 100, 50 and 10 mμ porosity.

Handling of specimens

Faecal specimens were collected by the nursing staff and were stored at $-20°$ C. while bacteriological tests were carried out. When no bacterial pathogens were isolated they were processed in the usual manner by preparing a 10 % suspension in cold nutrient broth containing 1000 units of penicillin and 1000 units of streptomycin per ml. and shaking in a McCartney bottle. The resulting supernatant, after three centrifugations at 3500 r.p.m. for 15 min., was used to inoculate tissue cultures.

Brief description of the outbreak

An outbreak of diarrhoea occurred in a section of an infants' home, Sydney, in April 1961. All the children in this section were below the age of 2 years. Upper respiratory tract infections and diarrhoea had occurred sporadically for about 6 weeks and at the time of collection of the specimens all children had diarrhoea. Faecal specimens from nineteen children were examined. Seven of the children had had an upper respiratory tract infection within the previous 7 days but the relationship of these symptoms to the virus isolated is not known.

RESULTS

Isolation and attempted serological identification of the virus

Of the nineteen specimens examined, fifteen yielded a virus cytopathic for H.Ep II cells but not for monkey kidney epithelium. Cultures of H.Ep II cells showed cytopathic effect 3–4 days after inoculation. Passage of the virus into further H.Ep II cells produced cytopathic effect in 48 hr. but passage into monkey kidney cultures still caused no degeneration.

It was thought that the virus was most likely to be a Coxsackie A 21 (Coe virus), but antisera to this virus failed to neutralize any of the viruses isolated and the antiserum prepared against the prototype Mill strain failed to neutralize 100 ID 50 Coe virus.

Similarly it was shown that the virus was not related to Coxsackie Group A viruses 11, 13, 15, 18 and 20, which are known to be cytopathic for HeLa and H.Ep II cells. A full study was then undertaken in an attempt to serologically identify the virus with members of the enterovirus group. Antisera to Polioviruses 1–3, Coxsackie B 1–6, Coxsackie A 1–10 and Echo viruses 1–28 all failed to neutralize the agent, as did antisera to Rhinoviruses F.E.B. and 1266 (a Rhinovirus isolated in this laboratory and cytopathic for H.Ep II cells). An antigen prepared from tissue culture fluids did not fix complement in the presence of five different sera known to contain Adenovirus complement-fixing antibodies, and Adenovirus antisera 1–17 and 19–24 failed to neutralize the virus.

The antiserum prepared against the Mill strain and possessing a homologous titre of 64 failed to neutralize 100 ID 50 of Coxsackie A viruses 12, 14, 16, 17, 19, 22 and 24 when tested in unweaned mice. It was therefore concluded that this virus was not antigenically related to any of the classified enteroviruses and did not possess the common soluble antigen of the adenovirus group.

Dr A. S. Abrahams has isolated a number of viruses with similar properties to the Mill strain and kindly sent four prototype strains and their homologous antisera. Cross-neutralization tests established the Mill virus as serologically indistinguishable from Hu 659, a virus isolated by Abrahams in Pittsburgh. No relationship to the other three viruses, Hu 39, Hu 2220 and C-Thai 18, could be demonstrated (Table 1).

The remaining fourteen strains were also neutralized by Mill and Hu 659 antisera.

Table 1. *Neutralization titres obtained with Mill virus and four strains received from Dr Abrahams*

	Virus	
Antiserum	Mill	Hu 659
Mill	64	64
Hu 39	< 4	< 4
Hu 659	128	128
Hu 2220	< 4	< 4
Thai 18	< 4	< 4

Cytopathogenicity of Mill virus in different tissue-culture systems

The Mill virus produced cytopathic changes in two continuous cell lines (HeLa and H.Ep II), human amnion, human embryonic fibroblasts and human embryonic kidney. It failed to produce any effect in monkey kidney, rabbit kidney, embryonic guinea-pig kidney, mouse embryonic fibroblasts and chick embryo fibroblasts.

The cytopathic effect seen in H.Ep II cells was similar to that shown by the cytopathic members of the Coxsackie A group. Small foci of refractile rounded or shrunken cells were first seen in the culture. These foci then spread throughout the culture until all cells were affected. In haematoxylin–eosin stained preparations the nucleus was frequently pyknotic and crescentic in shape and was usually located at the periphery of the shrunken cell.

The behaviour of this virus in human embryonic fibroblasts was unusual in that it was rapidly cytopathic when the growth medium consisted of Hanks's balanced salt solution, lactalbumin hydrolysate and calf serum, but it was not cytopathic when medium 199 was used. Alteration to the pH of medium 199 with dilute acid or alkali and variation in the bicarbonate concentration did not assist the virus to produce cytopathic changes. To date this unusual phenomenon has not been investigated further.

Inoculation of unweaned mice

Mill virus after three passages in H.Ep II cells failed to produce any obvious disease in mice inoculated when less than 24 hr. old and observed for 21 days. No lesions could be found in the brain, fatty tissue or skeletal muscle of mice killed at 7 and 14 days. The original faecal extracts from which the viruses were recovered also failed to produce any symptoms in unweaned mice. From these results it was concluded that the virus is not pathogenic for unweaned mice and therefore cannot be classified in the Coxsackie group.

Intraperitoneal inoculation of adult rabbits, guinea-pigs and mice failed to produce any symptoms. No pocks were found following inoculation of the chick embryo chorioallantoic membrane.

Haemagglutination

The virus was tested for haemagglutination by the method of Goldfield, Srihongse & Fox (1957) at 37, 22 and 4° C. No haemagglutination could be demonstrated using human O, rabbit, guinea-pig, mouse, fowl, calf or sheep erythrocytes.

Multiplication at 33° C.

When cultures of H.EpII cells, inoculated with Mill virus, were maintained at 33° C., both C.P.E. and virus titres were delayed by approximately 48 hr. as compared with cultures given a similar inoculum and maintained at 36° C. This suggests that the virus is not a member of the Rhinovirus group.

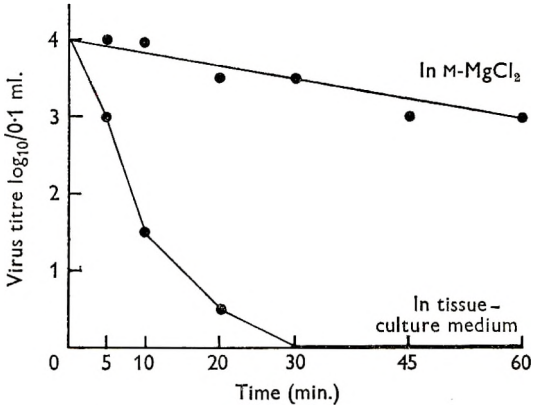

Fig. 1. Effect of heating virus at 50° C.

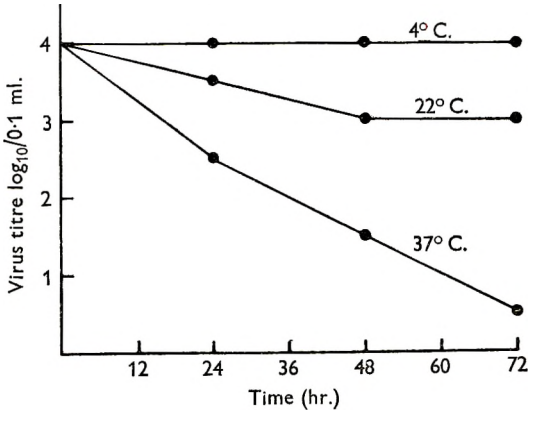

Fig. 2. Effect of temperature on virus survival.

Resistance to heat and chemicals

Equal volumes of virus suspension were heated at 50° C. in stoppered tubes for periods of up to one hour. No virus could be recovered after 30 min. at this temperature. Infectivity was retained, however, when the virus was heated at this temperature in the presence of M-$MgCl_2$ (Fig. 1). No loss of infectivity was found after storage at 4° C. for 72 hr., but 99 % of the virus was destroyed in 72 hr. at 37° C. (Fig. 2).

Resistance to ether is an important characteristic of enteroviruses and this was tested by adding one part of diethyl ether to four parts of virus suspension and gently agitating the mixture for 18 hr. at 4° C. The ether was then removed and the

virus titrated. The titre of the suspension before ether treatment was $10^{4.5}$ and afterwards $10^{3.5}$. The virus could therefore be said to be resistant to ether.

Treatment with HCl was used by Ketler, Hamparian & Hilleman (1962) to distinguish the Coryza–Rhinovirus group from the enteroviruses. The former were inactivated by the acid conditions but the latter survived. Mill virus after standing for 3 hr. at room temperature in HCl at pH 3 was not significantly reduced in titre.

Estimation of particle size

After treatment with fluorocarbon 113 the suspension was passed through Millipore membranes of 100, 50 and 10 mμ porosity. The virus was recovered from the first two filtrates but not the third. This gives an approximate size of 30 mμ, a figure generally accepted as the size of the enteroviruses.

Nature of the virus

Treatment of the cultures with 5-bromodeoxyuridine in concentrations of 10 and 50μg./ml. failed to prevent multiplication of the virus in H.Ep II cells, suggesting that it is a RNA virus. In parallel experiments vaccinia virus was completely inhibited.

The properties of Mill virus are summarized in Table 2. These properties suggest the virus should be classified in the enterovirus group.

Table 2. *Summary of properties of 'Mill' virus*

1. Size 30 mμ (approximately).
2. RNA virus.
3. Cytopathic in HeLa, H.Ep II and human amnion, embryonic kidney and fibroblasts.
4. *Not* cytopathic for monkey kidney, rabbit kidney, guinea-pig embryonic kidney and mouse or chick fibroblasts.
5. Serologically distinct from any of the classified enteroviruses or adenoviruses.
6. Not pathogenic for unweaned mice or adult mice, rabbits or guinea-pigs.
7. Does not produce pocks on the chick embryo chorioallantoic membrane.
8. Does not haemagglutinate human 'O' cells.
9. Resistant to ether, HCl at pH 3, and heating to 50° C. for 1 hr. in M-$MgCl_2$.
10. Inactivated at 50° C. for 1 hr. in T.C. medium.
11. Multiplication rate slower at 33° C. than at 36° C.

Antibodies in children from whom the virus was isolated

It was possible to obtain serum specimens from eight of the children from whom the virus was isolated and from another child in the home from whom an adenovirus only was isolated.

All eight children possessed neutralizing antibody to Mill virus but in low titres (8–32) only. Antibodies were not found in the serum from the other child. No antibodies could be found in a sample of human gamma-globulin.

There is no obvious explanation at present for the poor antibody response in the patients from whom the virus was isolated and also in the rabbits immunized with tissue-culture suspensions. It may be necessary to employ more sensitive methods using plaques to determine antibody titres as has been found in work with the Rhinoviruses.

SUMMARY

A virus was isolated from fifteen of nineteen children living in an infants' home during an outbreak of diarrhoea. The virus possesses many of the characteristics of the enterovirus group but is serologically distinct from any of the accepted members of this group. It is non-pathogenic for unweaned mice, non-cytopathic in tissue cultures of monkey kidney, but is rapidly cytopathic for tissue-cultured cells of human origin. It is serologically similar to virus Hu 659 isolated by Abrahams in the United States (personal communication).

I should like to thank the Director-General of Public Health and State Psychiatric Services, New South Wales, for permission to publish.

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Printed in Great Britain

An outbreak of myxomatosis caused by a moderately attenuated strain of myxoma virus

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(*Received* 20 *May* 1964)

INTRODUCTION

Since the original epizootic of myxomatosis in this country (1953–55) carcasses of rabbits from outbreaks of the disease have been sent regularly to the Central Veterinary Laboratory, Weybridge, but no detailed investigations have been carried out on outbreaks of disease in natural populations in Great Britain since 1954 (Armour & Thompson, 1955). In July 1962 a rabbit was sent from the East Riding of Yorkshire and as a matter of routine this establishment was informed of the outbreak. The population of rabbits concerned was large and occupied an area of approximately 200 acres. The strain of virus causing the outbreak was attenuated and when this was discovered efforts were made to secure samples for as long as the outbreak lasted.

MATERIALS AND METHODS

Carcasses were obtained from July 1962 until January 1963 at fortnightly intervals. The majority were shot or taken by dogs.

Rabbit fleas

In a disease the spread of which is influenced to a large extent by its vectors, it was thought advisable to examine the numbers of the principal vector (the rabbit flea—*Spilopsyllus cuniculi*) on individual rabbits.

Counts of fleas were made on rabbit carcasses which had been put into flea-proof linen bags immediately after killing.

Virulence tests

With a large initial population it was hoped that the outbreak would be sufficiently prolonged for the detection of any change in the virulence of the virus strain concerned. Samples of rabbits were obtained at fortnightly intervals and some of these were used for carrying out virulence tests.

These were carried out after the manner used in Australia by Fenner & Marshall (1957) and later in Great Britain by Chapple & Bowen (1963):

(*a*) *Rabbits.* New Zealand Whites weighing 4–4½ lb. kept in individual cages in a room with a constant temperature (60° F.).

* Present address: The World Health Organization, International Reference Centre for Respiratory Virus Diseases, Common Cold Research Unit, Salisbury, Wiltshire.

(*b*) *Virus preparation.* Virus was extracted by grinding lesion material with sterile sand in a cold pestle and mortar. The homogenate was suspended in a diluent containing phosphate buffer, skim milk, penicillin and streptomycin (Westwood, Phipps & Boulter, 1957). Sand and debris were removed by light centrifugation (2000 r.p.m. for 10 min.). The supernatants were stored at $-20°$ C. and titrated immediately before use.

(*c*) *Titration of virus preparations.* Virus suspensions were titrated on the chorio-allantoic membrane of 11-day-old chick embryos according to the technique of Westwood, Phipps & Boulter (1957).

(*d*) *Route of inoculation.* Groups of rabbits were inoculated intradermally into the shaved right flank with 0·1 ml. quantities of the virus suspensions. Five rabbits were used for each virus preparation. A record was kept of the clinical symptoms and survival times.

Gel diffusion precipitin reaction

We wanted to examine the usefulness of the gel diffusion technique in the study of myxomatosis. With this in view 124 rabbits were obtained during 3 consecutive days in September. Each rabbit was examined against positive antigen and anti-serum for the presence or absence of myxoma antigens and antibodies. From an economic point of view it was also important to know the recovery rate in the population.

The technique as described by Chapple, Bowen & Lewis (1963) was used. Pieces of lesion material, lung and blood clot were tested against known myxoma antigen and antiserum.

A watch was also kept on the behaviour of the disease and on any possible correlation between sex, disease incidence and recovery rate. A record was kept of the weight of each unpaunched carcass.

EXPERIMENTAL RESULTS

Field history and rapidity of spread

(Numbers in brackets refer to points on Fig. 1. The quotations are from the field officer's reports.)

January 1962—myxomatosis was seen near the railway line, but apparently died out. The field officer reported that twenty-four bodies were seen but that the disease was leaving 'far too many' apparently healthy rabbits.

March 1962—the disease was first officially observed at Jackdaw Plantation (1), but there was no evidence of spread. The farmer said he first saw it in this area about May.

2 July 1962—rabbit from point (2) examined by gel test in the laboratory with a positive result.

19 July 1962—three infected rabbits seen.

23 July 1962—four infected rabbits seen.

7 August 1962—'disease spreading' (but no bodies found).

13 August 1962—approximately twelve infected rabbits seen. Diseased rabbits seen in East Dale for the first time (3).

20 August 1962—'disease seems to have passed its peak as less infected rabbits and no bodies seen'.

21 August 1962—approximately twelve infected rabbits seen.

27 August 1962—approximately twelve infected rabbits seen.

On the first visit beyond the north-west end of Jackdaw Plantation (point 4) five infected rabbits were seen and disease may have been present there for some time.

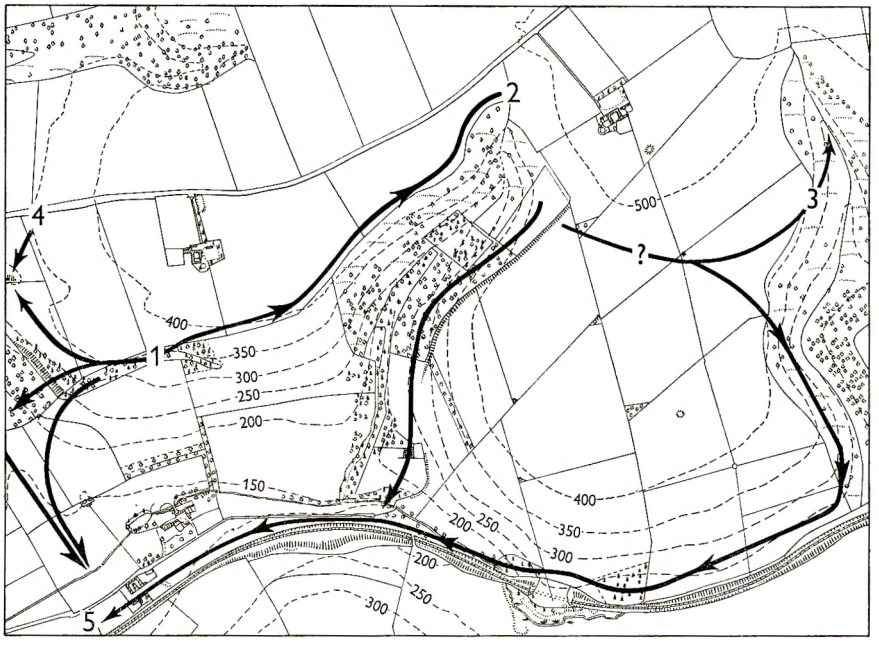

Fig. 1. Map showing St Austin's Dale, and direction of spread of outbreak. Scale, 3 in. = 1 mile. Reduced from the 6 in. = 1 mile ordnance survey map, by kind permission of H.M. Stationery Office.

3 September 1962—'disease position seems static'.

17–19 September 1962—sample of 124 rabbit carcasses obtained. The field officer considered that the peak infection was reached about 10 days before.

12 November 1962—disease thought to be approaching its peak in south-west corner (point 5).

10 December 1962—only one rabbit was seen and as a result the regular sampling of the population was terminated.

The field officer said he had not seen a 'typically' infected rabbit since the big sample. In fact the last was apparently obtained on 15 October. Others obtained were infected but seemed to be recovering.

On 6 March 1963 the keeper said that over the preceding 5 weeks he sold 234 rabbits in excellent condition. He also rejected some twelve or fifteen that showed symptoms of apparent recovery (one was sent to the laboratory on 6 March 1963). He also mentioned that three of the healthy ones contained 'a very hard ball-like object sort of gathered on the backbone which on being cut produced a white curd-like foul-smelling substance'. Presumably this was a chronic abscess produced

as a result of secondary infection of a regressing myxomatosis lesion. He also said at this time (6 March 1963) that the Jackdaw Plantation rabbits were all poor, undersized specimens though normally with no evidence of any disease.

Direction of spread

We have no knowledge of spread from the railway line and presumably the January outbreak died out there. According to the keeper it did not spread from Jackdaw Plantation. However, the farmer observed its spread along the edge of the dale to point (2), where a field officer first saw an infected rabbit.

From St Austin's Dale the disease was thought to have spread to East Dale (3). Throughout, all unwanted rabbits killed by dogs, shooting and by hand were buried in rabbit holes in the vicinity.

From East Dale, St Austin's Dale and Jackdaw Plantation the disease spread to the south-west corner of the estate (5)—on the site of the January outbreak. It seemed to be approaching a peak here on 12 November 1962.

Gel diffusion precipitin test

The studies on the precipitin reaction in agar gel have been reported in detail elsewhere (Chapple *et al.* 1963) and it is sufficient here to outline the results. Of the 124 rabbits examined there were twenty which appeared clinically healthy, showed no detectable antibody or antigen, and so were presumed to be susceptible to myxoma virus. A further group of fifteen apparently healthy rabbits could be divided into a group of ten which showed only antibody in the tissue examined and were therefore presumed recovered from infection; the remaining five showed the presence of antigen only and were presumed to be in the early stages of infection.

Of the rabbits which were clinically infected seventy-eight showed antigen, antigen and antibody or antibody in other tissues as well as blood. One had no antigen in its tissues and antibody was found only in the blood. One had neither antigen nor antibody in its tissues.

Table 1. *Summary of test results and clinical diagnosis*

(Based on Chapple *et al.* (1963), Tables 3 and 4.)

	Gel test diagnosis			
Clinical condition	Infected	Recovered	Susceptible	Total
Clean	5	10	20	35
Infected	78	1	1	80
Uncertain (?infected)	3	0	1	4
Uncertain (?clean)	1	0	2	3
Unknown	2	0	0	2

Another two groups with provisional clinical diagnoses (?infected, ?clean) were also examined, but the numbers were too small to draw any conclusion save that the gel test tended to confirm the provisional clinical diagnosis.

The remaining two rabbits' condition on receipt was such that a clinical diagnosis was not possible. The results are summarized in Table 1.

Table 2. *Summary of virulence tests on the St Austin's Dale and Devon Strains of myxoma*

	Virus	Date of isolation	No. of rabbits inoculated	Amount of virus inoculated (pock-forming units)	Time to primary lesion (days)	Time to secondary lesion (days)	Time to severe generalization (days)	Mean survival time (days)
				St Austin's Dale				
1	TPM/13/62	24.vii.62	5	30	2 (2)	4·8 (4–6)	6 (6)	16·6 (12–21)
2	TPM/15/62	24.vii.62	5	50	—	—	—	21·6 (14–28)
3	TPM/15/62	24.vii.62	5	15	NK	4·0 (4)	6·2 (6–7)	16·2 (11–22)
4	TPM/18/62	25.vii.62	4	10	NK	NK	NK	22 (21–23)
5	TPM/19/62	25.vii.62	4*	10	NK	NK	NK	22 (20–24)
6	TPM/26/62	8.viii.62	5	50	—	—	—	23·3 (19–27, 2S)†
7	TPM/34/62	22.viii.62	5	50	—	—	—	23·4 (19–34)
8	TPM/44/62	11.ix.62	5	50	—	—	—	25·4 (19–30)
9	TPM/149/62	18.ix.62	5	1,600	NK	4 (4)	6 (6)	19 (18–21)
10	TPM/175/62	25.ix.62	5	50	—	—	—	20·4 (17–23)
11	TPM/185/62	10.x.62	5	50	—	—	—	23·7 (17–33)
12	TPM/195/62	14.xi.62	5	100,000	NK	4·75 (4–5)	6 (6–7)	13·9 (11–16)
13	TPM/195/62	14.xi.62	5	50	—	—	—	22·3 (20–25, 1S)
				Devon				
	TPM/3/62	14.iv.62	5	—	2·2 (1–5)	6 (7–8)	8·2 (8–10)	18·8 (16–27)

* One died of pneumonia. † S = rabbit recovered from infection. NK, not known.

Attenuation of virus

In order that any attenuation of virus could be detected virulence tests were carried out using material obtained at intervals during the course of the disease. The first sample for such testing was obtained on 23 July 1962 and this gave a mean survival time (M.S.T.) of 16·6 (range 14–21) days. Samples were then tested which had been taken at fortnightly intervals over a period of approximately 4 months.

The results have been summarized in Table 2, and they indicate that no further attenuation of virus occurred during the period in question. Table 2 also illustrates the finding of Fenner & Marshall (1957) that quite large variations in the dose of virus make little difference to the survival times of the rabbits inoculated.

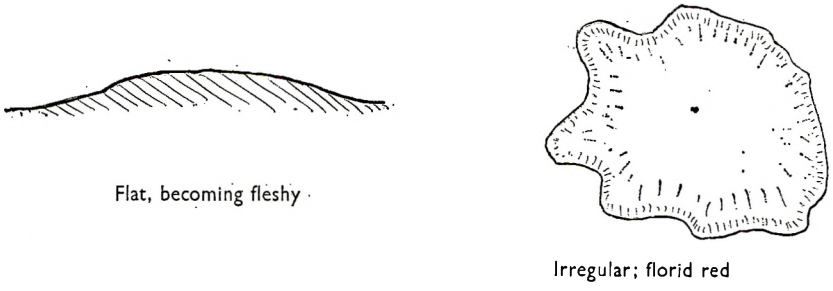

Fig. 2. Diagrammatic representation of the primary lesion caused by the St Austin's Dale myxoma virus strain.

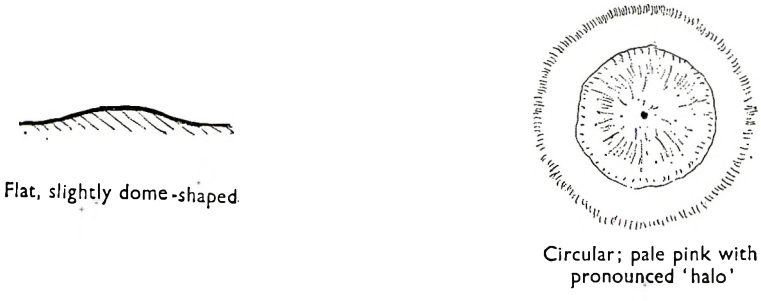

Fig. 3. Diagrammatic representation of the primary lesion caused by the Devon myxoma virus strain.

In all cases the primary lesion was large and irregular in outline with a florid red colour. In contour the primary lesion was flat at first, becoming rather raised and fleshy later. Because of the irregular shape it was not possible to obtain useful measurements of the lesions. A summary of the results of the virulence tests, including times for the onset of secondary lesions and for serious involvement of the head, is given in Table 2. Details of the Devon strain are also given in Table 2. Figs. 2 and 3 are diagrammatic representations of the differences in primary lesions of the St Austin's Dale and Devon strains of virus.

The clinical symptoms of the animals infected in the outbreak were indistinguishable from those observed during the epizootic of 1953–55.

Counts of fleas

Early in the course of the outbreak the field officers who were keeping watch on this rabbit population thought that the disease was not spreading as quickly as was usual in such a situation. It was suggested that the numbers of the principal vector (the rabbit flea) might not have been sufficiently large to initiate a rapid spread. To check this suggestion rabbit carcasses were obtained which had been put, immediately after killing, into flea-proof linen bags. Twenty-three rabbits were obtained in this way and 392 fleas were found, giving an average per rabbit of 17 (range 1–36). There were no rabbits completely devoid of fleas.

DISCUSSION

The area from which these rabbit samples were drawn is a part of the East Riding of Yorkshire known as St Austin's Dale, situated about 9 miles north-east of South Cave. The grid reference for the area is 92/93:33/35 and a map of the area (taken from 6 in. = 1 mile ordnance survey map by kind permission of H.M. Stationery Office) is shown in Fig. 1. This shows the large area of scrub in which the rabbit colony was based. The majority of the rabbits used the scrub for cover and the surrounding arable land as a source of food. The damage to the surrounding crops was considerable, some 5–10 acres of barley and about 5 acres of roots being rendered useless, besides damage to pasture. It is not possible to give an accurate estimate of the rabbit population at the beginning of the outbreak of myxomatosis. However, when the large sample of rabbits was obtained in September 1962 (approximately 6 months after the start of the outbreak) two independent observers counted 195 and 210 rabbits, respectively, on the combined west and north fringes of the dale. Even after the sample of 124 rabbits was obtained the numbers were not markedly diminished. Taking into consideration the time the disease had been present, the amount of damage, the effect of obtaining the large sample and the counts made, the initial population was estimated to be in the region of 1000 rabbits.

The mean survival times of all the samples, except one, are of the same order and it was thought that all showed the same strain of virus and that no further attenuation had occurred. The sample which gave a mean survival time of 13·9 days was retested using a smaller inoculum (50 instead of 10^5 p.f.u.) and the result indicates that this sample was not significantly different from the other samples. In the light of Fenner & Marshall's (1957) evidence that quite large variations in dosage do not make great differences to the survival times, an additional explanation must be sought. The experiment in question was carried out in the winter of 1962–63 when air temperatures were extremely low. Although there was heating in the animal house the temperature outside dropped sufficiently to cause the freezing of the water supply. Previous experience has shown that environmental temperature can have a profound effect on survival times (Mykytowycz, 1956; Fenner & Marshall, 1957; Chapple & Bowen, 1963). We feel that the mean survival time of 13·9 days could have been produced as a result of both the large dosage of virus and the drop in environmental temperature.

From the rabbits obtained there is the suggestion that there was approximately an 8% recovery rate (92% mortality) which accords quite well with the recovery rate found with a virus giving a mean survival time somewhere between 16 and 28 days (Fenner & Marshall, 1957). We do not claim the sample of 124 rabbits to be random. It is more than likely that there was a higher percentage of recovered rabbits in the population, as it is much easier to catch or shoot a relatively immobile diseased rabbit. Making an approximate estimation of the recovery rate of 10% this means that a nucleus of 100 rabbits would be left out of the estimated population of 1000 plus any rabbits which the disease had by-passed. This nucleus would rapidly build up to the original numbers if undisturbed by ordinary control measures. In fact, on a day in June 1963, 293 rabbits were counted and the report added the comment that this number of rabbits obviously represented only a fraction of the total population.

Table 3. *Correlation of sex, infection and recovery rates of* 106 *rabbits where it was possible to determine sex*

Sex	Totals	Recovered (AB only)	Susceptible (no AG or AB)	Infected (AG or AG+AB)	AG only	AG+AB
M	47	11	14	22	7	15
F	59	7	7	45	15	30

The significance of the rabbit flea in myxomatosis has been the subject of some discussion (Rothschild, 1960; Andrewes, Thompson & Mansi, 1959) and much of this discussion has been based upon the supposition that the rabbit flea is a sedentary ectoparasite. However, recent work by Mead-Briggs (1964) has shown that this vector is more mobile than was hitherto supposed and we have concluded that the numbers of fleas found on the rabbits (av. 17 per rabbit) from this area would be quite sufficient to promote efficient spread of the disease.

An attempt was made to correlate sex, disease incidence and recovery rate and Table 3 shows the relationship of the presence or absence of antigen and antibody with sex. Although these figures suggest that males have less chance of infection and that if infected they have a greater chance of recovery, the numbers of rabbits involved are too small for any definite conclusions to be drawn.

The record of the weights showed a relatively normal distribution for the time of year, the type of site and method of collection. The sex ratio is also normal for such a sample.

Since the detailed records kept of the original outbreak in the Edenbridge area of Kent, no comparable records have been made of other outbreaks and so our knowledge of the behaviour of attenuated strains of myxoma virus in the field, in this country, is negligible.

Before any conclusions can be reached on the evolution of myxoma virus in Great Britain further detailed studies, combining proper field observations with full laboratory tests, must be made. Such studies are not as difficult as they were 5 years ago when the rabbit population was very low. Since that time certain areas,

especially those where rabbits are difficult to control (e.g. cliffs, quarries, War Department ranges, and those areas where scrub is maintained for game rearing purposes) have suffered a resurgence of the rabbit followed by periodic outbreaks of myxomatosis. It is these areas which are now the potential experimental sites.

We should like to record our gratitude to those members of the Ministry of Agriculture's Staff at Leeds and Beverley, Messrs M. M. Senior, S. P. Clark, S. Charlton and K. S. Boden, who made this study possible. A very special word of thanks is due to Mr R. A. R. Matthews who collected most of the rabbit specimens. We are grateful to the owner of the land for permission to collect samples and for allowing the many visits of the local Ministry of Agriculture staff.

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J. Hyg., Camb. (1964), **62**, 443
Printed in Great Britain

The campaign against typhoid and paratyphoid B in western Norway. Results of cholecystectomy

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(*Received* 19 *May* 1964)

INTRODUCTION

At the beginning of this century a considerable number of typhoid and paratyphoid B cases were notified every year in western Norway. The figures also remained high during the First World War and the following years. However, since 1922 there has been a uniform decline, and in recent years only a few cases have been notified.

The distribution of the two diseases has been exceedingly uneven in the various districts in western Norway. Such distribution is a cogent argument for the opinion that the infection is conveyed by chronic carriers. The first chronic carrier in western Norway was detected in 1918 (Madsen, 1918). In 1920 the guiding principles were formulated for the institution of a systematic campaign against enteric diseases in western Norway. In particular, great pains have been taken to discover the original sources of infection in the form of chronic carriers. Since 1918, 147 individuals have been found to be chronic carriers, 84 being carriers of *Salmonella typhi*, and 63 of *Salmonella paratyphi B*. The chronic carriers of older date discovered by systematic searching for the source of infection among the contacts of new cases of typhoid, numbered 91. Among these chronic carriers were 37 who had previously suffered from enteric fever. Twelve of these were first found to be carriers more than 20 years after infection, and several of them were not found to be carriers till after the age of 70. Cases kept under constant control from the time of their illness and found to discharge the specific organisms after recovery from it, numbered 39. Three were found to be carriers following bacteriological examination of gall-bladders after their removal on account of attacks of biliary colic. On systematic bacteriological examination of the gall-bladder carried out at all the post-mortem examinations conducted at The Gade Institute, 14 individuals were found to have been carriers. In only 2 of these cases had there been a history of enteric fever.

Of the 147 chronic carriers, 23 were males and 124 females. Thus, in this material women were much in the majority, accounting for 84 % of the chronic carriers. Most of the carriers were elderly folk, 96 (65 %) being over the age of 50 when their carrier state was discovered. There were 37 carriers over the age of 70 at this stage. Prigge (1912) associated the high chronic carrier rate in elderly persons with the incidence of gall-stones, which also rises with age. The post-mortem material from the Bergen City Hospital for the period 1912–28 (Vogelsang, 1929), consisting of

2690 individuals over the age of 20, showed a gall-stone rate of 6·5 %, 3·9 % for men and 9·8 % for women. There was a considerable rise in this rate among women around the age of 50. A similar rise came at a higher age in men. It is therefore possible that the greater liability of elderly women to suffer from gall-stones contributes to the frequency with which they become chronic carriers.

From time to time attempts have been made to cure the chronic state by various means. Occasional cures have been reported as a result of various procedures, but further investigations have shown them to be ineffective in most cases. As the carrier state in the majority of cases depends on processes in the gall-bladder, we have tried to cure the carriers by operation.

OPERATION ON CHRONIC CARRIERS

Among the 147 chronic carriers who discharged the specific organism in the faeces, there were 14 in whom this condition was not discovered till a post-mortem examination was made. Among the remaining 133 there were 66 on whom operations were performed with a view to curing the carrier state.

Cholecystotomy

In 2 cases, only cholecystotomy could be performed. In a 50-year-old workman's wife the operation was limited to cholecystotomy because of the state of her gall-bladder, which contained pus and 12–14 stones of the size of a walnut. There was also an angular stone as large as a pea in the much thickened cystic duct. *S. paratyphi B* was found in the biliary fistula up to 4 days after the operation. All the later bacteriological examinations gave negative results. She was considered cured.

The other, a 58-year-old farmer, stated that he had suffered from typhoid 40 years earlier. As the position of the gall-bladder was abnormal and because it was firmly adherent to the common bile duct and portal vein, only cholecystotomy was performed. The biliary fistula took 2 months to close, and discharge of *S. typhi* in the faeces continued.

Thus, in 1 of the 2 cases in which cholecystotomy was performed, a cure of the carrier state was effected, while it persisted in the other case.

Cholecystectomy

The gall-bladders of 64 chronic typhoid and paratyphoid B carriers were removed by operation with the results given in Table 1. Two died as a result of the operation, 9 remained carriers, 53 are considered cured. A distinction can be made between the two infections, but this does not demonstrate any striking difference in the results achieved following operation in chronic typhoid or chronic paratyphoid B carriers.

Two deaths and 9 failures to achieve sterilization may seem to bulk large in relation to so small a total as 64 cases. On the other hand, the 64 cases included 53 cures. The operation was therefore successful in four-fifths of the cases.

Two deaths must be related to the operation. A 56-year-old farmer's wife was

detected as a chronic carrier $7\frac{1}{2}$ years after she had suffered from typhoid. She was examined in the Bergen City Hospital and, as she had myocarditis, she was advised against operation. However, she herself wished to have the operation, and a cholecystectomy was carried out at her urgent request in another hospital. She died of heart failure 10 days after the operation.

Table 1. *Results of cholecystectomy*

Specific organism	Died	Remained carrier	Considered cured	Total
S. typhi	1	6	31	38
S. paratyphi B	1	3	22	26
Total	2	9	53	64

A technical mistake was made during the operation on an insane woman who was operated on at the age of 39, $1\frac{3}{4}$ years after she had suffered from paratyphoid B. Her death from cholaemia 6 days after the operation was caused by the common bile duct having been divided and ligated about 8 cm. above the papilla of Vater.

CHRONIC CARRIERS WITH NORMAL STERILE GALL-BLADDERS

The gall-bladders of two individuals who remained carriers were found at the operation to be perfectly normal, without stone formation. Microscopical examinations showed no pathological changes in the walls of the gall-bladders, and bacteriological examinations showed the contents of the gall-bladders to be sterile. Both these carriers continued to discharge *S. typhi* with the faeces after the operation. As in both cases the gall-bladder was found to be macroscopically and microscopically normal, to contain sterile bile and no stones, it seems unlikely that the focus of infection was situated higher up in the bile passages or in the liver. As, however, the duodenal contents continued to contain *S. typhi* after the operation, it seems natural to suspect a focus of infection in the neighbourhood of the duodenum. As the canal of Wirsung opens into the common bile duct below the cystic duct, it is conceivable that these 2 carriers continued to discharge *S. typhi* because of a focus of infection in this canal or in the pancreas. On X-ray examination, no pancreatic stones were found. As both carriers are still alive, though very old women, the seat of infection in both these cases must remain in doubt for the present.

CONTINUED DISCHARGE OF THE SPECIFIC GERM AFTER OPERATION

A female shop assistant was 24 years old when operated on, $1\frac{1}{2}$ years after she had suffered from paratyphoid B. A biliary fistula communicating with the exterior remained open. She died from tuberculosis $1\frac{1}{2}$ years after the operation without being rid of *S. paratyphi B*, which was found in the liver together with tuberculous necrosis.

Of the other 6 carriers who continued to discharge the specific germ after operation, the time of infection is unknown in one (Table 2). In the other 5 the operation

was carried out 4 and 5 months, 1 year, 17 and 46 years respectively, after infection. The age at the time of the operation was in 1 case 30 years, in 4 cases between 50 and 60, and in 1 case 63 years. The carrier who was operated on 5 months after infection died of a cerebral tumour 7 years after the operation. At the post-mortem examination *S. paratyphi B* was cultivated from the common bile duct and the hepatic duct. It therefore seems that the specific process may in a few cases be situated at a higher level in the bile passages of the liver, as well as in the gall-bladder.

Table 2. *Interval between infection and operation*

Interval	Died	Remained carrier	Considered cured	Total
3 months	—	—	1	1
4 months	—	1	1	2
5 months	—	1	2	3
½ year	—	—	4	4
¾ year	—	—	2	2
1 year	—	1	6	7
1½–2 years	1	1	2	4
2–3 years	—	—	3	3
3 years	—	—	1	1
4–5 years	—	1	1	2
5–10 years	1	—	2	3
10–15 years	—	1	1	2
15–20 years	—	1	3	4
30–39 years	—	—	1	1
40–49 years	—	1	3	4
50 years	—	—	2	2
Unknown	—	1	18	19
	2	9	53	64

In typhoid fever a histological examination reveals necrotic foci in the liver. Bile congestion may easily arise as a result of inanition, meteorism and the like, and it may well be that a focus formed in the liver may provide favourable conditions for the further growth of the organisms.

CRITERIA OF CURE

The number of bacteriological examinations of chronic carriers after operation has varied with the duration of excretion of the organisms. Though some carriers were found to be negative at every examination after operation, others were found to harbour the specific organism for varying intervals of time after operation before the carrier state ceased.

Bacteriological examination usually started from 4 to 6 days after operation and continued at intervals of a few days. In 14 patients only faeces were examined, and in most of these the operation was performed before the introduction of duodenal intubation as a routine procedure. No carrier was regarded as cured until he had had at least five consecutive negative faecal tests; in fact there were only 2 carriers who did not have more than this minimum number.

Otherwise, we have attached much greater importance to a negative duodenal

test than to a negative faecal test. Since the introduction of duodenal intubation as a routine procedure after the operation, we have required a minimum of three consecutive negative faecal tests and one negative duodenal test; there were, however, only six carriers who did not have more than this minimum number of negative results.

Table 3. *Numbers of consecutive negative examinations after operation*

No. of negative tests			
Faeces	Duodenal contents	No. of carriers	
5	—	2	
6	—	4	
7	—	2	
8	—	2	14
9	—	1	
10	—	2	
22	—	1	
3	1	6	
4	1	3	
5	1	2	
6	1	3	
7	1	2	
11	1	1	
13	1	1	
26	1	1	
3	2	3	
4	2	3	39
5	2	2	
6	2	4	
7	2	2	
9	2	1	
10	2	1	
13	2	1	
14	2	1	
7	4	1	
6	5	1	

Sixteen carriers showed only negative results after operation. In the other carriers the consecutive negative results recorded in Table 3 followed some positive results. It was particularly in the carriers whose first tests after operation were positive that more than one negative duodenal test was required before the carrier was considered cured. In most of these carriers the faeces had become negative within the 1st month after operation, but the last two in Table 3 showed positive faecal results over a considerable period. For this reason these two carriers were not considered cured until they had had four and five negative duodenal tests respectively in addition to the negative faecal tests.

Although in certain patients it might have been desirable to undertake further tests, most of them had so many consecutive negative results that the chances of our being mistaken in regarding them as cured are very small indeed. It is now many years since several of these carriers were operated on, and in the interval no case of enteric fever has occurred which could be connected with any of them.

INDICATIONS FOR OPERATION

The experiences provided by the present material point to the following indications for operation and the time at which it should be undertaken:

The specific organism must be demonstrable in the bile by duodenal intubation.

If, at the same time, cholecystography indicates stones in the gall-bladder, the operation should be carried out when the carrier state has lasted at least 3 months after the end of the illness.

If X-ray examinations indicate only functional disturbances of the gall-bladder, the operation ought not to be carried out less than 6 months after the end of the illness.

If the functional activity of the gall-bladder is shown on X-ray examination to be normal, it is as well to be reserved in recommending operation.

Most individuals must experience quite a severe physical shock when told they are chronic carriers. Because of this state, many of them cannot continue in their former occupation, and the most conscientious of them are in constant terror of infecting those about them. A few of our carriers have committed suicide. With the growing realization by the public that removal of the gall-bladder offers chances of a cure of the carrier state, there will be a growing demand by chronic carriers for this remedy, which, as matters are at the present time, would seem to be difficult to prevent.

In recent years several investigators have tried to cure the carrier state with antibiotics. Further investigations are needed here. However, it is to be hoped that we shall soon have in our hands an antibiotic or other drug which is excreted by the liver in such concentrations that it will be able to kill the specific organisms and thereby cure the carrier state of these really unhappy individuals.

SUMMARY

1. The first chronic carrier in western Norway was discovered in 1918. Since then 147 individuals have been found to be chronic carriers, 84 being carriers of *S. typhi* and 63 of *S. paratyphi B*.

2. Of the 147 chronic carriers 23 were males and 124 females. Most of the carriers were elderly folk, 96 over the age of 50 and 37 over the age of 70 when their carrier state was discovered. There is a considerable rise in the incidence of gall-stones among women around the age of 50, while a similar rise comes at a higher age in men. This sex difference may be a contributory cause of the larger number of female carriers.

3. Operations were performed on 66 chronic carriers with a view to curing the carrier state. In 2 cases, only cystotomy could be performed because of the state of the gall-bladder. In one of these the carrier state was cured, while it persisted in the other.

4. The gall-bladders of 64 chronic carriers were removed by operation with the following results: 2 died as a result of the operation, 9 remained carriers, and 53 are considered cured. The operation was therefore successful in four-fifths of the cases.

The gall-bladders of 2 individuals who remained carriers were found at operation

to be sterile and perfectly normal, without stone formation. Two other individuals who remained carriers have later died, $1\frac{1}{2}$ and 7 years after their operations. In both cases *S. paratyphi B* was cultivated from the common bile duct and the hepatic duct. The specific process may therefore in a few cases be situated at a higher level in the bile passages of the liver.

5. The interval between infection and operation varied from 3 months to 50 years. The 2 carriers who had the longest interval are both cured.

6. Indications for operation are given on the basis of the experiences provided by the present material.

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J. Hyg., Camb. (1964), **62**, 451
Printed in Great Britain

The epidemiology of leptospirosis in North Queensland

I. General survey of animal hosts

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INTRODUCTION

The east coast of North Queensland (Fig. 1) differs from the rest of the Australian continent in combining high rainfall with tropical temperatures. Leptospirosis was first recognized here in 1933 in the sugar-cane fields of Ingham (18° 39′ S., 146° 10′ E.) by Morrissey (1934), and the laboratory investigations were reported by Cotter & Sawers (1934). Lumley (1937) named the first two serotypes isolated, and three others had been identified (Johnson, 1950) by the time the Institute's Field Station was established at Innisfail (17° 32′ S., 146° 02′ E.) in 1951. Subsequent investigation of cases of fever increased the number of serotypes known to infect man in the area to fourteen (Sinnamon *et al.* 1953; Smith *et al.* 1954; Smith & Brown, 1955; Broom & Smith, 1956; Addamiano, Babudieri & Smith, 1960; Alexander & Smith, 1962), and their general epidemiology was analysed by Derrick *et al.* (1954) and Derrick (1956). A fifteenth (*Leptospira bindjei*) has been recognized since our work was completed, and is reported in Part II (Battey, Smith & Barrow, 1964).

The association of rodents and marsupial bandicoots with canefield leptospirosis was observed in the early investigations (Cotter & Sawers, 1934). Leptospires seen in the kidneys of bandicoots were not isolated and identified, but *Leptospira zanoni* (= *australis B*) was recovered from *Rattus rattus*, and *L. australis* (= *australis A*) was isolated in the Ingham district from *R. sordidus conatus*, which was recorded at that time as *R. culmorum* (Cotter, 1935; Sawers, 1938). A later series of animals was reported by Doherty, Emanuel & Moore (1956), and is included in the present survey. These authors found that 11 out of 16 *R. s. conatus* from the Babinda and Mirriwinni areas (Fig. 1) were infected with *L. australis*, nine being urinary carriers, and that antibodies to *L. australis* and other serotypes were present in the sera of bandicoots.

Nearly half the cases of leptospirosis in the area proved to have no occupational relation to the canefields (Derrick, 1956), and it soon became apparent that a knowledge of the associations between the serotypes and their animal hosts was essential before the epidemiology of the disease could be fully understood. There

was a further incentive to pursue this line of inquiry. Leptospirosis causes an appreciable amount of illness and economic loss (largely in payment of compensation for lost wages) in North Queensland, but it is rarely fatal, and people do not fear it enough to exert themselves seriously to prevent it. Burning the cane in the

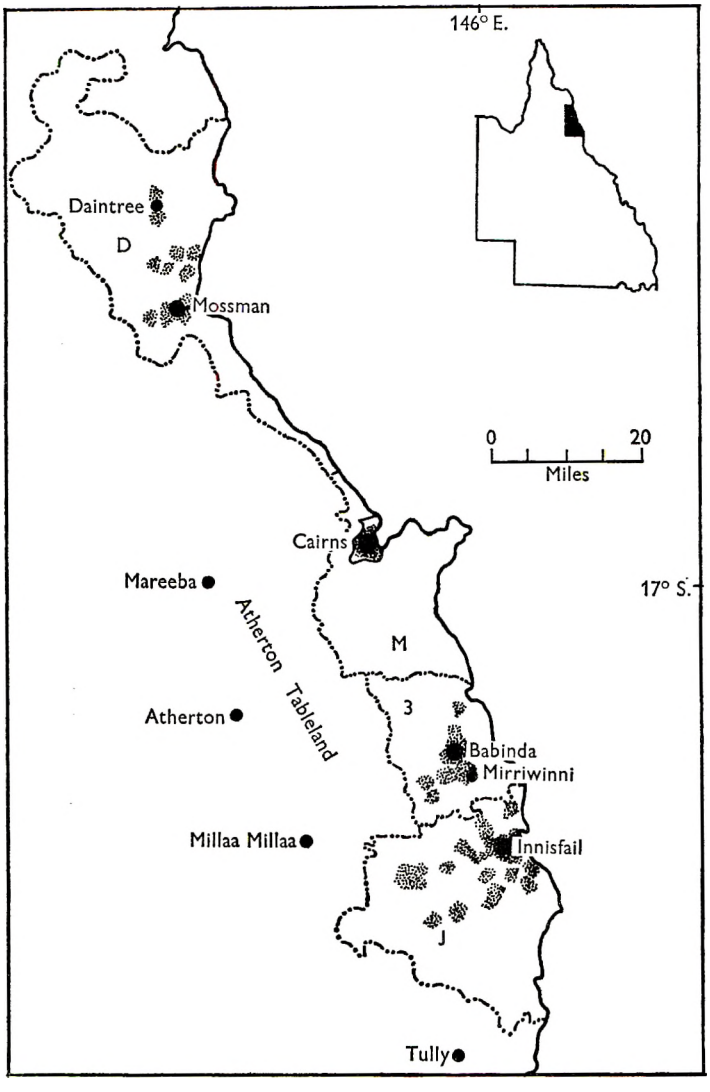

Fig. 1. Map showing trapping areas (stippled) in North Queensland. Shires are indicated by: J, Johnstone; D, Douglas; 3, Division 3 of M, Mulgrave.

fields before it is cut is the only community measure that can be enforced, and it eliminates neither rats nor leptospires (Emanuel & Harrison, 1961; Doherty *et al.* 1956); protective clothing will not be worn; while vaccination, if it were effective, would meet apathy and possibly obstruction. It is difficult to escape the conclusion that the only method of prevention that might be socially and economically practicable would be some form of ecological control, and that could not even be

thought about without a clear understanding of the hosts, their ecology, and the conditions which determine the incidence of infection in them.

This phase of the research was therefore developed in two stages. In the first, which is reported here and in Part II, the plan was to examine as many animals of as many species as possible, over a relatively wide range of country, in an attempt to assess the significance of different species as hosts of different serotypes. The second stage grew out of the ecological studies of the mammals, which proceeded concurrently (Harrison, 1962). Natural leptospiral infections were followed in the course of mark–recapture observations on the animals, and information was obtained on duration of infection, persistence of antibodies, and the relationship between density of host populations and the incidence of infection in them. These data are still being analysed.

MATERIALS AND METHODS

Most of the material in the general survey was collected by the staff of the Field Station between June 1953 and May 1961. It comprised 5 monotremes, 463 marsupials, 1350 rodents, 67 bats, 295 domestic mammals, 30 birds, 28 reptiles, and 21 amphibians. It has been supplemented, where appropriate (notably in Tables 3 and 4), by records from an additional 180 bandicoots and 1005 rodents that were collected in the mark–recapture areas.

Collection of animals

The topography of the area has been described by Derrick *et al.* (1954) and Harrison (1962). It presents a series of mountain ranges and tablelands, separated from the Pacific Ocean by a narrow coastal plain. Except for a lower range running approximately parallel to the coast, the land between the mountain chain and the coast is used mainly for growing sugar cane.

Most of the wild animals examined were obtained from the Johnstone, Mulgrave (Division 3), and Douglas shires, including the towns of Innisfail, Babinda, and Mossman (Fig. 1). The average annual rainfall in these areas varies from approximately 90–150 in. In addition, 28 animals from Cairns were examined, and 61 sera from the Atherton Tableland, including 46 samples from marsupials collected in 1945 for Professor F. J. Fenner.

The types of trap used at the Field Station allowed small mammals to be caught alive. Local residents trapped most of those from the Mulgrave shire and several from other areas. In the earlier work, an attempt was made to trap areas in which human infections had occurred, generally in or close to canefields.

An outbreak of leptospirosis in dairy cattle was investigated in conjunction with veterinary officers of the Queensland Department of Agriculture and Stock. Additional cattle sera, pig sera, and a few sheep sera were obtained from abattoirs or from veterinarians, and specimens from wild pigs were collected by men on hunting parties.

Methods of examination

Wild animals were given serial numbers, and species, sex, sometimes weight, and usually whether the animal was young or mature, were recorded. The animals were

killed by ether or chloroform, pinned out, and swabbed with a 1:1000 solution of 'Zephiran'. After dissection of the chest wall, heart blood was taken by sterile syringe or Pasteur pipette, and allowed to clot. The abdomen was dissected, and small pieces of kidney cortex were placed in culture media, using fresh sterile instruments for each stage of the dissection.

Kidneys, and sometimes other tissues, were cultured in 2·5 ml. quantities of Schüffner's and Fletcher's media containing 20 % rabbit serum dispensed in ¼ oz. McCartney bottles. The cultures were incubated at 30° C., and were examined at intervals for 28 days. When leptospires were seen, subcultures were made.

Material for dark-ground examination was obtained by scraping the cut surface of the kidney with a sterile platinum loop, and rubbing up the material in one or two drops of saline on a glass slide. A 22 mm. square coverslip was applied, and the whole area searched for leptospires, using an 8 mm. objective. Freshly voided urine was examined similarly when it was not desired to kill the animal.

Tissues from some wild animals were inoculated into laboratory mice. Quantities of 0·5 or 1 ml. of emulsion of kidney alone, or a mixture of liver, spleen and kidney, were inoculated intraperitoneally into each of a group of two or four mice. Guinea-pigs were occasionally used in addition to mice.

Sera only were obtained from most of the domestic animals. All blood specimens were allowed to clot at room temperature, and, after separation, the serum was stored in the refrigerator until transported to Brisbane.

Serology and identification of strains

Agglutination tests on the sera and identification of the leptospires isolated were carried out at the Laboratory of Microbiology and Pathology, Brisbane, by the methods described by Smith *et al.* (1954) and Smith & Brown (1955); except that the technique of the cross-absorption test was changed during the latter half of the investigation, when formalized suspensions were substituted for suspensions of living organisms, and serum was absorbed for 18 hr. at 30° C. Thirteen serotypes reported from man in North Queensland were used as antigens in the agglutination test: *L. icterohaemorrhagiae*, *L. canicola*, *L. zanoni*, *L. robinsoni*, *L. australis*, *L. bratislava* (= *esposito* of earlier publications), *L. pomona*, *L. grippotyphosa*, *L. medanensis*, *L. kremastos*, *L. mini*, *L. hyos*, and *L. celledoni*. In addition, most of the sera were tested against *L. autumnalis*, and some against *L. bataviae*. *L. cynopteri* was included in the tests on some bat sera.

Interpretation of serological results

Tabulation of the serological results from the animals from which leptospires were isolated showed that most serotypes normally produced a characteristic pattern of serological response which was independent of the host species. However, there was sometimes appreciable overlap, so the unsupported serological findings must be interpreted with caution. Thus, other serotypes sometimes induced production of heterologous antibodies to *L. icterohaemorrhagiae* to such high titres that it would be unwise to identify this serotype on serological evidence alone;

therefore it and its possible hosts are recorded with question marks in the tables. The serological response to infection also fails to differentiate adequately between *L. zanoni* and *L. robinsoni*, between *L. australis* and *L. bratislava*, between *L. medanensis*, *L. kremastos* and *L. mini*, and between *L. canicola*, *L. broomi* and *L. bindjei*. Reactions to these serotypes will therefore usually be referred to as *pyrogenes*, *australis*, *hebdomadis*, and *canicola* serogroups, respectively.

In the absence of proof by isolation or dark-ground observations, titres of 1:100, with normal spread of reactions, have been accepted as reasonable evidence of past infection in wild animals. This policy is conservative, but analysis of the titres recorded in infections proved by isolation of the organism suggests that the incidence of infection was not seriously underestimated. Concurrent high titres to serotypes that do not usually overlap were accepted as evidence of more than one past infection. Antibody patterns in the rodents indicated relatively few multiple infections, but reactions to two or more unrelated serogroups were demonstrated in 28 % of the infected bandicoots.

Interpretation of the antibody patterns in the domestic animals is more difficult. Titres of 1:100 are recorded, but possibly antibody levels only of 1:1000 or higher in cattle and pigs should be accepted as reliable evidence of infection. The scatter of positive reactions at serum dilutions of 1:100 and less, without the clear patterns generally found in the wild animals, may be due to previous leptospiral infections, but it raises the suspicion that the findings may be non-specific. Thus, Babudieri & Gaspardis (1959) suggested that the agglutination reactions found in bovine sera may sometimes be paraspecific, and Schebitz & Dedié (1955) found that non-specific stimuli may stimulate or increase antibody formation against leptospires in horses.

Comparison of survey methods

Table 1 shows the percentage of infected animals according to species in 1498 rodents and bandicoots which were examined by all three methods of kidney culture, dark-ground examination, and serology.

Dark-ground and culture. Overall results show that dark-ground examination was inferior to cultivation for the detection of kidney infections. Of the animals in Table 1, leptospires were seen on dark-ground examination in 53 (3·5 %), and were grown in culture from 74 (5 %). The number of animals showing kidney infections by one or both methods was 86 (5·7 %). Dark-ground examination was especially useful in the series of *R. assimilis*, in which no successful culture was obtained from four of the animals in which leptospires had been observed in the kidneys.

Animal inoculation. Material from relatively few wild animals was inoculated into laboratory mice and guinea-pigs. However, this procedure increased the number of strains isolated from 74 to 77. One strain of *L. celledoni* from *R. assimilis* was isolated from the kidneys of an inoculated laboratory mouse, and two strains of *L. australis* in *R. s. conatus* from a mouse and a guinea-pig respectively. In all three wild animals, leptospires were observed on dark-ground examination of the kidney.

Demonstration of leptospires and serology. Of 1498 animals in Table 1, leptospires

were demonstrated by one or both methods of culture and dark-ground examination in 86 (5·7 %), and antibody titres of 1:100 or more were recorded in 311 (21 %). The total number of animals showing infections, as determined by either demonstration of leptospires or antibody titres of 1:100 or more, was 328 (22 %).

Table 1. *Comparison of survey methods in bandicoots and rodents examined by all three methods*

Species	No. examined	Infection rates determined by: Culture (%)	Dark-ground (%)	Serology (%)	All methods (%)
R. norvegicus	13	61	23	46	69
P. nasuta	48	10	2	60	60
I. macrourus	325	2	0·6	45	45
R. s. conatus	108	18	19	30	33
H. chrysogaster	48	0	0	25	25
M. musculus	130	16	16	9	17
R. assimilis	137	0·7	4	13	14
R. rattus	411	3	0·2	12	12
U. caudimaculatus	26	0	0	8	8
Melomys spp.	252	0·8	0	1	1
Total	1498	5·0	3·5	21	22
		5·7			

Serological results for the 86 animals in which leptospires were demonstrated show that 69 (80 %) had agglutination titres of 1:100 or more. The percentage of serologically positive animals was appreciably different from the total percentage of infected animals in only two species, *R. norvegicus* and *Mus musculus* (Table 1). Five out of eight *R. norvegicus* in which leptospires were demonstrated showed titres of 1:100 or more, one showed a titre of 1:30, and two 1:10 against *L. zanoni*. Eleven out of 21 *M. musculus* excreting *L. zanoni* showed titres of 1:100 or more, nine showed titres of 1:30, and one showed a titre of 1:10 to the homologous serotype. In addition, the following animals in which leptospires were demonstrated showed antibody titres less than 1:100 against the homologous serotype: two *R. s. conatus* with no detectable antibodies to *L. australis*, one *R. s. conatus* with a titre of 1:10 to *L. zanoni*, one *R. assimilis* with a titre of 1:30 to *L. celledoni*, and one *R. assimilis* with none to *L. hyos* (but 1:1000 to *L. pomona*).

INFECTIONS IN MARSUPIALS AND RODENTS

The species examined

Bandicoots and rodents are the most abundant mammals in the area, and they received most attention, the traps used being particularly adapted to capture them. Consequently, there is some bias against the larger or arboreal marsupials, which do not appear to be particularly numerous in the coastal strip, but they too were examined whenever an opportunity arose. Brief notes on the species are given below, and a more detailed account of them can be found in the paper by Harrison (1962), whose nomenclature is followed here.

Marsupials

Antechinus flavipes godmani (Thomas), marsupial mouse. Insectivorous; confined to rain forest.

Perameles nasuta Geoffroy, long-nosed bandicoot. Common in vegetation of all types; an animal about a foot long, which hunts and roots for its insect food, sleeping in a nest of a heap of leaves.

Isoodon macrourus (Gould), short-nosed bandicoot. Similar to *P. nasuta*, but usually confined to cultivated areas or secondary woodland, where it is very numerous.

Trichosurus vulpecula (Kerr), common brush-tailed possum. Arboreal; specimens from open eucalypt forest have been examined, but none of the rain-forest form.

Hypsiprymnodon moschatus Ramsay, musky rat-kangaroo. An insectivorous animal of the floor of rain forest; rather like a bandicoot.

Aepyprymnus rufescens (Gray), rufous rat-kangaroo. A small, kangaroo-like animal of the open eucalypt forest.

Dendrolagus lumholtzi Collett, Lumholtz's tree kangaroo. A rain-forest species adapted for tree climbing.

Thylogale stigmatica Gould, red-legged pademelon or scrub wallaby. A wallaby (i.e. a small kangaroo) of the floor of rain forest.

Protemnodon agilis (Gould), wallaby. An open-country species occurring in the cultivated areas.

Rodents

Hydromys chrysogaster Geoffroy, water rat. A large, carnivorous species associated with watercourses, but ranging far afield.

Melomys cervinipes (Gould), naked-tailed rat. A climbing rat of rain forest and woodland generally.

M. lutillus littoralis (Lönnberg), naked-tailed rat. Very similar to the last, and often difficult to distinguish from it, but smaller, and confined to grassland, becoming a pest of sugar cane.

Uromys caudimaculatus (Krefft), giant naked-tailed rat. A very large, climbing rat of rain forest.

Rattus assimilis (Gould), allied rat. A moderately large, native rat of the floor of rain forest, eating both insects and vegetable matter.

R. sordidus conatus Thomas, canefield rat. A native rat of grassland or open forest; here found almost exclusively in canefields.

R. rattus (Linnaeus), house rat. Common in houses and gardens, and occurring also in cultivated fields and the edges of woodland.

R. norvegicus (Berkenhout), Norway or brown rat. A cosmopolitan house rat; confined in northern Queensland to the centres of coastal towns.

Mus musculus Linn., house mouse. Common in houses, and occurring also in gardens and cultivated fields.

Table 2. *Leptospiral infections in marsupials and rodents—general survey*

Species	No. examined	Total animals infected	Serogroups	No. of infections	
				Total	Urinary
Marsupials					
Perameles nasuta	67	34	*?icterohaemorrhagiae*	1	—
			canicola	1	—
			pyrogenes	10	—
			australis	15	—
			pomona	1	—
			hebdomadis	14	5
			hyos	3	—
			celledoni	4	—
Isoodon macrourus	362	150	*canicola*	29	2
			pyrogenes	56	1
			australis	47	1
			pomona	19	—
			hebdomadis	36	2
			celledoni	4	1
Antechinus flavipes godmani	5	0	—	—	—
Trichosurus vulpecula	15	1	*hebdomadis*	1	—
Aepyprymnus rufescens	6	0	—	—	—
Hypsiprymnodon moschatus	2	0	—	—	—
Dendrolagus lumholtzi	1	0	—	—	—
Thylogale stigmatica	2	1	*grippotyphosa*	1	—
Protemnodon agilis	3	0	—	—	—
Rodents					
Hydromys chrysogaster	68	15	*?icterohaemorrhagiae*	1	—
			pyrogenes	5	—
			australis	7	—
			hyos	3	—
Uromys caudimaculatus	41	3	*pyrogenes*	2	—
			australis	1	—
			hyos	1	—
Melomys cervinipes	80	1	*celledoni*	1	1
Melomys lutillus	248	2	*canicola*	1	1
			australis	1	—
			celledoni	1	—
Rattus sordidus conatus	122	37	*pyrogenes*	5	2
			australis	34	22
Rattus assimilis	157	21	*?icterohaemorrhagiae*	1	—
			pyrogenes	3	—
			australis	1	—
			pomona	12	3
			hyos	6	2
			celledoni	1	1
Rattus rattus	471	51	*?icterohaemorrhagiae*	2	—
			canicola	1	1
			pyrogenes	29	12
			australis	12	—
			pomona	8	—
			grippotyphosa	3	—
Rattus norvegicus	13	9	*pyrogenes*	9	8
Mus musculus	150	22	*pyrogenes*	21	21
			australis	4	1

Host distribution of the serotypes

It is necessary to distinguish between the broader results that were derived principally from serology, and the more precise ones that were obtained when the leptospires were isolated and identified. The serogroups recorded from the 1813 marsupials and rodents examined in the survey series are set out in Table 2. Leptospiral infections were detected in all nine species of rodents and the two bandicoots in sufficient numbers to indicate that these small, predominantly terrestrial, fossicking animals were reservoirs of at least some representatives of all the major serogroups known from North Queensland. Antibodies were found also in two other marsupials (at 1:300 to the *hebdomadis* serogroup in a *Trichosurus vulpecula* from the Atherton Tableland, and at 1:100 to *L. grippotyphosa* in a *Thyogale stigmatica* from near Innisfail), but their significance is not so clear.

Table 3. *Leptospires isolated from bandicoots and rodents*

Serotypes	Numbers of strains	Hosts
L. broomi	3	*I. macrourus* (2) *R. rattus* (1)
L. bindjei	1	*M. lutillus* (1)
L. zanoni	47	*I. macrourus* (3) *M. lutillus* (1) *R. s. conatus* (2) *R. assimilis* (2) *R. rattus* (12) *R. norvegicus* (7) *M. musculus* (20)
L. robinsoni	2	*U. caudimaculatus* (1) *R. s. conatus* (1)
L. australis	39	*P. nasuta* (2) *I. macrourus* (1) *R. assimilis* (5) *R. s. conatus* (29) *R. rattus* (1) *M. musculus* (1)
L. kremastos	5	*P. nasuta* (4) *I. macrourus* (1)
L. mini	1	*I. macrourus* (1)
L. hyos	4	*U. caudimaculatus* (1) *R. assimilis* (3)
L. celledoni	3	*I. macrourus* (1) *M. cervinipes* (1) *R. assimilis* (1)

The information about the 105 strains of leptospires that have been identified is given in Table 3, which is set out differently from Table 2, because it includes isolations made in the mark–recapture experiments as well as from the survey series. There is also a series of urinary infections in *R. assimilis* from which the organisms have not been isolated. Most of these were encountered in a mark–

recapture experiment in rain forest. Sera from the rats reacted strongly with *L. pomona*, and the organisms from the urine of several were transmitted to laboratory mice, which also developed antibodies to *L. pomona*, but all attempts to recover them in culture have failed. Presumably they are either a mutant, which has lost its capacity to grow in Schüffner's and Fletcher's media, or a closely related serotype.

Most of the identifications of the commoner serotypes were from the survey series, so it follows that the infections recorded in Table 2 on serological grounds as *pyrogenes* serogroup would have been caused predominantly by *L. zanoni*, and those recorded as *australis* serogroup predominantly by *L. australis*. The hosts of *L. broomi* are also indicated fairly clearly by the isolations. The *pomona*, *grippotyphosa*, *hyos*, and *celledoni* serogroups are each represented in Australia by a single serotype, so reactions to them may be accepted as specific, with the reservation that there may still be undiscovered variants or near relatives.

The errors introduced into the analysis by accepting these seven specific identifications seem likely to be small, but the remaining serogroups cannot be treated in the same way. Comparison of our findings with Derrick's (1956) analysis of frequency of occurrence and association with canefields suggests that *I. macrourus* may prove to be a better host for *L. mini* than *P. nasuta*, and that *U. caudimaculatus* and *R. s. conatus* are probably not the only hosts of *L. robinsoni*. It may be inferred too, by exclusion, that bandicoots are probably the hosts of *L. canicola*. We have only the sketchiest indication of possible hosts of *L. icterohaemorrhagiae*, and none at all of those of *L. bratislava*.

Thus, there is evidence that nearly all of the serotypes that have been isolated from man in the area occur also in this section of the animal population. Conversely, no serotype that is not known from the human population was recovered from the animals. Few serological tests were done with representatives of serogroups that have not yet been recorded in Australia, but cultures were made from more than 1600 of the animals in this series, so any undetected serotypes would have been uncommon or ill-adapted to grow on the media used.

Multiple infections

The numbers of infections recorded in Table 2 exceed the numbers of infected animals, reflecting the fact that some animals had had more than one infection. It is difficult to recognize concurrent infections with two or more serotypes (Smith & Doherty, 1956; Alexander *et al.* 1957). Two (both with *L. australis* + *L. hyos* in *R. assimilis*) have been demonstrated by isolation in the mark–recapture experiment referred to above, but none in the survey series, and it is not possible to indicate whether the infections with different serogroups recorded on serological grounds in the following animals were concurrent or successive:

In 13 *P. nasuta:* 1 *?icterohaemorrhagiae* + *australis*, 1 *canicola* + *australis*, 2 *pyrogenes* + *australis*, 1 *pyrogenes* + *hebdomadis*, 1 *pyrogenes* + *celledoni*, 4 *australis* + *hebdomadis*, 1 *australis* + *hyos*, 1 *pyrogenes* + *australis* + *hebdomadis*, 1 *hebdomadis* + *hyos* + *celledoni*.

In 39 *I. macrourus*: 4 *canicola* + *australis*, 2 *canicola* + *hebdomadis*, 12 *pyrogenes* + *australis*, 2 *pyrogenes* + *pomona*, 10 *pyrogenes* + *hebdomadis*, 2 *australis* + *hebdomadis*, 1 *australis* + *celledoni*, 1 *pomona* + *hebdomadis*, 1 *pomona* + *celledoni*, 2 *hebdomadis* + *celledoni*, 2 *pyrogenes* + *australis* + *hebdomadis*.

In 1 *H. chrysogaster*: *pyrogenes* + *australis*.

In 1 *U. caudimaculatus*: *pyrogenes* + *australis*.

In 1 *M. lutillus*: *canicola* + *celledoni*.

In 3 *R. assimilis*: *pyrogenes* + *pomona*, *australis* + *hyos*, *pomona* + *hyos*.

In 4 *R. rattus*: 3 *pyrogenes* + *australis*, *pomona* + *grippotyphosa*.

In 3 *M. musculus*: *pyrogenes* + *australis*.

The numbers of multiple infections in most species are too small for statistical tests, and all that can be said is that they are of the order to be expected if the chances of infection with different serogroups are independent of one another. With *I. macrourus*, numbers are large enough to show that, while this is true of most multiple infections, the numbers with *pyrogenes* + *australis* serogroups ($\chi^2 = 8{\cdot}4$, $P < 0{\cdot}01$) and with *pyrogenes* + *hebdomadis* serogroups ($\chi^2 = 10{\cdot}0$, $P < 0{\cdot}01$) are significantly in excess of chance expectation, but not the numbers with *australis* + *hebdomadis* serogroups. This is a curious finding that is difficult to interpret; it may be a manifestation of focality.

Maintaining and incidental hosts

Audy (1958) has developed the important concept of maintaining hosts, which ensure the perpetuation of a particular local population of parasites without the intervention of other, incidental hosts. The qualities that distinguish the two classes will be discussed later; we are concerned here only to find out whether they can be recognized in the associations that we have studied.

It is evident, from Table 2, that one species of mammal may be host to more than one serotype, and that individual serotypes may infect more than one species of host. There are, however, some definite associations, which become clearer when the numbers of infections are reduced to rates per 100 animals of the species, and the data are arranged in a form to facilitate comparison, as in Table 4, which includes infections recorded at first examination of mark–recapture animals as well as those in the survey series. The frequency with which leptospires are excreted in the urine is of crucial importance in transmission and maintenance, so this information is included also, isolation from the kidneys being regarded equally with actual detection of leptospires as an indicator of urinary excretion.

The frequency of excretion can be used to provide two different kinds of information, and two different ratios are therefore defined. One is the excretion rate, which is the number of urinary excretors per 100 of the whole population examined; it is shown, for the associations in which it can be calculated, as the left-hand figure in each column in Table 4. The other is the *excretion index*, which is the ratio of the number of animals excreting leptospires to the total number known to have been infected; it is expressed as a decimal fraction rather than a percentage, because a different notation should reduce the risk of confusion between the two ratios.

The most striking association observed was that between the *hebdomadis* sero-

Table 4. *Frequency of leptospiral infections per hundred animals in the wild populations*

Species	No. examined	(*?icterohaemorrhagiae*)		*canicola* serogroup		*pyrogenes* serogroup		*australis* serogroup		*pomona*		*grippotyphosa*		*hebdomadis* serogroup		*hyos*		*celledoni*	
		L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T
P. nasuta	124	—	2	—	0·8	—	12	2	18	—	0·8	—	—	**4**	**16**	—	3	—	3
I. macrourus	485	—	—	0·4	6	0·4	14	0·2	11	—	4	—	—	**0·6**	**8**	—	—	0·2	0·8
H. chrysogaster	92	—	1	—	—	—	8	—	8	—	—	—	—	—	—	—	4	—	—
U. caudimaculatus	83	—	—	—	—	1	5	—	5	—	2	—	—	—	—	1	4	—	—
M. cervinipes	233	—	—	—	—	—	—	—	—	0·5	2	—	—	—	—	—	—	0·5	0·5
M. lutillus	520	—	—	0·2	0·2	0·2	0·6	—	2	—	—	—	—	—	—	—	—	—	0·2
R. s. conatus	433	—	—	—	—	2	4	**12**	**19**	—	—	—	—	—	—	—	—	—	—
R. assimilis	306	—	0·3	—	—	0·3	2	2	4	**4**	**12**	—	—	—	0·6	1	3	0·3	0·3
R. rattus	520	—	0·4	0·2	0·2	**3**	**7**	0·8	5	—	1	—	0·6	—	—	—	—	—	—
R. norvegicus	13	—	—	—	—	**61**	**69**	—	—	—	—	—	—	—	—	—	—	—	—
M. musculus	155	—	—	—	—	**13**	**13**	0·6	2	—	—	—	—	—	—	—	—	—	—

L, Leptospires seen or isolated; T, total infections.
The most clearly defined associations are shown in bold type.

group and the marsupials, particularly the bandicoots, which showed a combined excretion index of 0·14 (609 examined, 59 infected, 8 excreting). No rodents were infected with this serogroup in the survey series, but two *R. assimilis* with titres of 1:100 were encountered in a mark–recapture experiment. Infections with the *canicola* serogroup were also significantly more frequent in the bandicoots than in the rodents ($\chi^2 = 107{\cdot}6, P < 0{\cdot}01$). The excretion index was 0·07 in the bandicoots, but it might have been higher for *L. broomi* alone, and the picture is somewhat obscured by the possibility that *L. bindjei* may be maintained by *Melomys*.

The introduced rodents are the principal hosts of *L. zanoni* in the sample, the frequency of excretion of leptospires being significantly higher in them than in any other species ($P < 0{\cdot}01$). Among the native rats, *R. s. conatus* stands out as the principal host of *L. australis*, total and excretion rates being significantly higher than in any other species ($P < 0{\cdot}01$ for both rates), while the excretion index (0·6) is notably high. The figures are, to an extent, inflated by inclusion of a heavily infected series collected during an outbreak of leptospirosis near Babinda (Doherty *et al.* 1956); nevertheless, they confirm an association that has been known since leptospirosis was first recognized in this country. That *R. s. conatus* may not be the only maintaining host of *L. australis* is indicated by the discovery of an apparently self-contained focus of infection in *R. assimilis* in the rain-forest experiment already mentioned. An even clearer rain-forest association, however, is that between *L. pomona* (or a variant) and *R. assimilis*, with an infection rate of 12 % and an excretion index of 0·3.

These associations have all been encountered so much more frequently than would have been expected by chance that the species or groups of animals involved may be presumed, at least provisionally, to be maintaining hosts for the particular serotypes. The situation is not so clear for two other predominantly rain-forest infections. Both *L. hyos* and *L. celledoni* are rather evenly distributed among their host species, and the principal suggestions of maintenance come from their relatively high excretion indices.

Many of the infections included in Table 4 do not fit into the patterns described above. They are scattered among the host species, usually in relatively small numbers, and they generally show a low excretion index, which suggests that most of them are transitory. We would regard these as probably representing casual infections in incidental hosts. Thus, both bandicoots, *R. rattus*, and *R. assimilis* seem to be able to acquire casual infections with most of the serotypes that they do not normally maintain, and the large rats, *H. chrysogaster* and *U. caudimaculatus*, appear to serve as incidental hosts for a more limited range of serotypes rather than as maintaining hosts for any. It is more difficult to decide whether *R. s. conatus* is an incidental or focal maintaining host for *L. zanoni* and *M. musculus* for *L. australis*, and it seems likely that the status of some of the species may vary with local circumstances.

Geographical distribution of infections

Derrick (1956) analysed 219 cases of leptospirosis in man diagnosed in North Queensland, of which two were from the Atherton Tableland and 188 from the

coastal shires of Johnstone, Mulgrave, and Douglas, within which he treated Division 3 of Mulgrave (Fig. 1) as a separate area. The incidence of leptospirosis was significantly higher, and infections with *L. zanoni*, *L. australis*, and *L. hyos* significantly more frequent, in Mulgrave 3 than elsewhere; *L. australis* preponderated significantly over other local serotypes in Mulgrave 3, and *L. zanoni* in Johnstone; and *L. medanensis* was found only in the northern part of the coastal strip, *L. robinsoni* in the southern. We have attempted a similar analysis of the infections listed in Table 2.

Atherton Tableland. This area lies at 2000–3000 ft., has a rainfall less than half that of the coastal strip, and is used mainly for dairying and growing tobacco. Derrick recorded *L. zanoni* and *L. hyos*, and we have found antibodies to *L. hyos* in *U. caudimaculatus* and *R. assimilis*, to *L. celledoni* in *P. nasuta*, and to the *hebdomadis* serogroup in *T. vulpecula*, as well as to *L. pomona* and *L. hyos* in cattle and pigs. All that can be said of the area is that at least five serotypes can survive there.

Table 5. *Relative frequency (per cent) of infections in bandicoots and rodents in the geographical divisions*

Serogroups	Johnstone, 200 infections in 1117 animals	Mulgrave 3, 162 infections in 282 animals	Douglas, 51 infections in 255 animals
icterohaemorrhagiae	?2	?0·5	?2
canicola	0	18	6
pyrogenes	50	22	8
australis	24	41	14
pomona	6	0	53
grippotyphosa	0	0	6
hebdomadis	11	15	8
hyos	5	0·5	0
celledoni	2	3	4

The coastal strip. Dissection of the records showed variations in total incidenc of infection of the same order as those found by Derrick, the relative proportions in the Johnstone, Mulgrave 3, and Douglas areas being 1:3·3:1·2 for the animals and 1:4·4:1·9 for man. The incidence of infection in bandicoots was also significantly higher in Mulgrave 3 than in the other areas ($\chi^2 = 21{\cdot}1$, $P < 0{\cdot}01$), and in *R. s. conatus* significantly higher in Mulgrave 3 than in Johnstone ($\chi^2 = 26{\cdot}2$, $P < 0{\cdot}01$). The relative frequency of the serotypes within each area is shown in Table 5. As in man, the *pyrogenes* group predominated in Johnstone and the *australis* group in Mulgrave 3, and the only notable discordance is a high incidence of *L. pomona* (in *I. macrourus* and *R. rattus*) in Douglas. When the species were examined separately, the only other striking difference observed was in *R. rattus*, which showed *L. zanoni*, *L. australis*, *L. pomona*, and ?*L. icterohaemorrhagiae* infections in Johnstone, *L. broomi* and *L. australis* in Mulgrave 3, and *L. pomona* and *L. grippotyphosa* in Douglas.

Population studies have not been undertaken outside the Johnstone shire, and no reason can be suggested for the differences that have been observed between the

three coastal areas. The points of immediate relevance that emerge from the study are that the infections in man and the animals show a degree of parallelism that is compatible with an association between them, and that the maintaining hosts, at least of *L. zanoni*, may not be the same in all parts of the coastal strip.

Infections in town, field, and forest

Urban infections. Infections in towns can be divorced from those in adjacent canefields only in Cairns (population 21,000) and Innisfail (7000). Derrick recorded 1 infection with *L. celledoni* from Cairns, and 4 with *L. zanoni* from Innisfail. Five *I. macrourus*, 1 *H. chrysogaster*, 21 *R. rattus*, and 1 *M. musculus* were examined from Cairns; all were negative. The Innisfail series comprised 9 *P. nasuta*, 13 *I. macrourus*, 16 *H. chrysogaster*, 1 *M. lutillus*, 3 *R. assimilis*, 104 *R. rattus*, 13 *R. norvegicus*, and 7 *M. musculus*. Evidence of infection with *L. zanoni* was found in 3 *P. nasuta*, 6 *I. macrourus*, 1 *H. chrysogaster*, 9 *R. rattus*, 9 *R. norvegicus*, and 1 *M. musculus*, while 2 *R. rattus* might have been infected with *L. icterohaemorrhagiae*. In one small area along the river bank, infections were found in 8 out of 8 *R. norvegicus* (7 excreting), in 3 out of 25 *R. rattus* (1 excreting), and in 1 *I. macrourus*. No other serotypes were detected in these animals, and thus the area apparently provided a pure focus of infection with *L. zanoni*. It is not surprising that infections have occurred in the human population.

Rural infections. The coastal area is dominated by rain forest on the hills and sugar cane on the plains, and each form of vegetation has its own characteristic fauna, which, excluding stray animals, is listed below (Harrison, 1962).

	Canefields	Rain forest
Marsupials	*I. macrourus* *P. nasuta*, less frequent	*P. nasuta* (3 other species of minor importance)
Rodents	*M. lutillus* *R. s. conatus* *R. rattus* *M. musculus*	*M. cervinipes* *R. assimilis* *U. caudimaculatus*
	H. chrysogaster in wet places anywhere	

Other forms of vegetation, such as secondary forest, grazing land, and swamp, constitute a minor part of the vegetation pattern, and the species found there also inhabit canefields or rain forest, depending on propinquity.

Derrick (1956) examined the relationship of the infections in man to occupation in canefields, and his findings can be summarized in the following statements, omitting *L. icterohaemorrhagiae*, *L. pomona*, and *L. medanensis*, for which there were insufficient data:

Positively associated with canefields: *L. australis*, *L. mini*.

Negatively associated with canefields: *canicola* serogroup.

Without predominance in or away from canefields: *L. zanoni*, *L. robinsoni*, *L. kremastos*, *L. hyos*, *L. celledoni*.

By reason of their wider distribution but more limited range of movement, the small ground mammals are better indicators than infected people of the places in

Table 6. *Infections in rodents and bandicoots from different habitats*

Species	Canefields						Rain forest						Other habitats						
	No. examined	No. of infections					No. examined	No. of infections					No. examined	No. of infections					
		zanoni	*robinsoni*	*australis*	*pomona*	*hebdomadis* gp.	*hyos*	*zanoni*	*australis*	*pomona*	*hebdomadis* gp.	*hyos*	*celledoni*		*?icterohaem-orrhagiae*	*zanoni*	*australis*	*pomona*	*hebdomadis* gp.	*hyos*	*celledoni*
P. nasuta	22	4	—	6	—	7	—	13	—	2	1	4	2	1	8	—	1	1	—	1	—	—
I. macrourus	62	12	—	6	—	8	—	0	—	—	—	—	—	—	26	—	2	—	—	2	—	—
H. chrysogaster	21	2	—	4	—	—	1	3	1	—	—	—	—	—	5	—	—	—	—	—	2	—
U. caudimaculatus	0	—	—	—	—	—	—	10	1	1	—	—	—	—	26	—	1	—	—	—	—	—
M. cervinipes	5	—	—	—	—	—	—	48	—	—	—	—	—	1	21	—	—	—	—	—	—	—
M. lutillus	99	—	—	1	—	—	—	0	—	—	—	—	—	—	70	—	—	—	—	—	—	—
R. assimilis	3	—	—	—	—	—	—	86	1	—	10	—	1	—	47	1	2	1	1	—	4	1
R. s. conatus	83	4	1	11	—	—	—	0	—	—	—	—	—	—	3	—	—	—	—	—	—	—
R. rattus	174	18	—	11	1	—	—	0	—	—	—	—	—	—	43	—	2	—	—	—	—	—
M. musculus	127	20	—	4	—	—	—	0	—	—	—	—	—	—	6	—	—	—	—	—	—	—

which populations of leptospires are maintained. The records for the Johnstone shire are divided according to habitat in Table 6, from which it is apparent that there is a wider range of serotypes in each of the rural habitats than in the urban infections recorded earlier, and that the 'other habitats' served, in general, as areas of overflow from the canefields or rain forest for the leptospires as well as for the mammals.

L. zanoni (60 infections), *L. australis* (43), and *hebdomadis* serogroup (15) were the commonest leptospires in the canefields, as is also indicated by the records of their occurrence on eight widely scattered cane farms in the district, namely: *L. australis* in 8, *L. zanoni* in 6, *hebdomadis* serogroup in 5. When the figures were corrected for uneven sampling, it was clear that *R. s. conatus* predominated among the ground rats and *L. australis* among the serotypes on most of the farms sampled; but both the populations of particular species and the incidence of infection varied considerably, and Table 7 illustrates a situation in which *M. musculus* and *L. zanoni* had been unusually abundant. It is also evident, from all the data, that *L. zanoni* was more strongly associated with canefields in this district than Derrick's figures would have suggested.

Table 7. *Infections found on one cane farm*

Species	No. examined	No. infected	Serotypes	No. of infections	
				Urinary	Total
I. macrourus	13	5	*zanoni*	—	2
			australis	—	3
			mini or *kremastos*	—	1
R. s. conatus	26	7	*zanoni*	1	1
			australis	4	6
R. rattus	41	2	*zanoni*	—	1
			australis	—	1
M. musculus	92	17	*zanoni*	16	16
			australis	1	3
Melomys spp.	4	0	—	—	—

L. pomona (11 infections) and the *hebdomadis* serogroup (4), associated respectively with *R. assimilis* and *P. nasuta*, were the commonest leptospires in the rain-forest animals. A similar occurrence of *L. hyos* and *L. celledoni*, with *H. chrysogaster* and *M. cervinipes* added to the hosts, is less apparent, because 6 of the infections were in animals collected in gallery forest along small water courses, and they consequently appear under 'other habitats' in Table 6. The mark–recapture experiment to which several references have already been made has shown that a focus of infection with *L. pomona*, and probably *L. australis* and *L. hyos*, was being maintained in rain forest by *R. assimilis*, while the *hebdomadis* serogroup was being maintained by *P. nasuta*, which was also serving as an incidental host of other serotypes. In this experiment, 40 out of 103 *R. assimilis* were found to be infected at first examination, an incidence that would rival that in *R. s. conatus* in heavily infected canefields.

OTHER WILD ANIMALS EXAMINED

Leptospires have been isolated from bats in Indonesia (Alston & Broom, 1958) and aquatic birds in Italy (Babudieri, 1958), and serological evidence of infection has been found in reptiles in Roumania (Combiesco *et al.* 1959) and Malaya (Gordon Smith, Turner, Harrison & Broom, 1961*a*), including a high incidence in the file snake (*Acrochordus javanicus*), which occurs also in Australia. A systematic survey of these groups was not attempted in the present study, but any individuals that were collected were examined. Sera were tested from nearly all, but dark-ground examinations and cultures from only about half.

Table 8. *Wild animals, other than marsupials and rodents, examined for leptospiral infections*

Species	No. exmd.	Species	No. exmd.
Monotremes		Birds (*cont.*)	
Ornithorhynchus anatinus (Shaw)	1	*Centropus phasianinus* (Latham)	4
Tachyglossus aculeatus (Shaw)	4	*Pitta versicolor* Swainson	1
Bats		*Cracticus quoyi* (Lesson & Garnot)	12
Pteropus conspicillatus Gould	33*	*Acridotheres tristis* Linnaeus	1
Pteropus alecto gouldii Peters	1	Reptiles	
Rhinolophus megaphyllus Gray	12	*Liasis childreni* Gray	1
Hipposideros bicolor (Temminck)	5	*Boiga fusca* (Gray)	3
Nyctinomus loriae Thomas	1	*Acrochordus granulatus* (Schneider)	2
Miniopterus schreibersi (Kuhl)	8	*Stegonotus plumbeus* (Macleay)	1
Miniopterus australis Tomes	5	*Natrix mairii* (Gray)	1
Nyctophilus bifax Thomas	2	*Oxyuranus scutellatus* Peters	1
Birds		*Denisonia* sp.	1
Rallus pectoralis Temminck	1	*Laticauda* sp.	1
Hypotaenidia philippensis (Linnaeus)	3	*Varanus tristis orientalis* Fry	8
Rallina tricolor Gray	2	*Tiliqua scincoides* (Shaw)	2
Amaurornis ruficrissus (Gould)	1	*Tiliqua gerrardii* (Gray)	2
Lobibyx sp.	1	*Egernia major* (Gray)	3
Nettapus pulchellus Gould	1	*Emydura latisternum* (Gray)	1
Accipiter fasciatus (Vigors & Horsfield)	1	*Crocodilus johnsoni* Krefft	1
Podargus strigoides (Latham)	1	Amphibians	
Halcyon macleayi Jardine & Selby	1	*Bufo marinus* (Linnaeus)	21

* Antibodies at 1:100 or more to *L. australis* in 6.

The species examined are listed in Table 8. Of the monotremes, the platypus (*O. anatinus*) is confined to rivers and burrows in their banks, and the echidna is terrestrial, itinerant, and lives on ants. The first two bats listed are fruit-bats or 'flying-foxes'; the others are all insectivorous, and some of them have a wide distribution outside Australia. The birds are a miscellaneous lot, some of which were caught in traps set for the small mammals. The reptiles include representatives of four families of snakes, two lizards, one freshwater tortoise, and one freshwater crocodile. The toads (*B. marinus*) were introduced into Queensland to control sugar-cane grubs, and are now extremely abundant.

No leptospires were isolated from any of these animals, and antibodies were detected only in the flying-foxes (*P. conspicillatus*).

INFECTIONS IN DOMESTIC MAMMALS

The coastal plain in North Queensland, in which nearly all the human cases of leptospirosis originated, and from which most of the wild animals in the survey were obtained, carries relatively few domestic stock. Dairy farming is, however, a major industry on the slopes to the west of this area, on the Atherton Tableland, and in the north of the Douglas shire (Fig. 1). Pigs are also raised on many of the farms. Beef cattle predominate in the drier areas of the Atherton Tableland and west to the Gulf of Carpentaria. Domestic dogs and cats are everywhere.

These animals are relevant to the present investigation in two ways: they may be primary reservoirs, as is well known for *L. canicola* in dogs in other parts of the world (Alston & Broom, 1958) and for *L. pomona* and *L. hyos* in pigs and cattle in southern Queensland (Johnson, 1950); or they may serve as incidental hosts, providing a possible link in a chain of infection from wild animals to man. Infections in cattle may also present a significant economic problem in Queensland (Sutherland, Simmons & Kenny, 1952), and an episode of this kind is described below.

Leptospirosis in a dairy herd

An outbreak of illness and abortions occurred in a dairy herd in the Palmerston section of the Johnstone shire, and veterinary officers made a provisional diagnosis of leptospirosis. Blood samples were taken from thirty-six cows, one calf, and one horse, and urine samples from two cows and the calf. At that time, six of the seven cows which had not been in calf when the outbreak began had been ill, sixteen of the others had aborted, and thirteen were still pregnant. Subsequently, six of the pregnant cows aborted and five calved (with the death of one calf, apparently from leptospirosis); the histories of the other two were not followed. Eighteen of the cows had antibodies to *L. pomona* at titres of 1:1000 or more, four had titres of 1:300 or 1:100, four 1:30, and two 1:10. Leptospires were observed in the urine of one cow which had an antibody titre of 1:3000. The calf had been very ill: leptospires were observed in its urine, and its serum had a titre of 1:30,000 to *L. pomona*.

The horse showed titres of 1:100 or more to the following serotypes: *L. icterohaemorrhagiae* 1:300, *L. australis* 1:300, *L. bratislava* 1:10,000, *L. pomona* 1:30,000, *L. grippotyphosa* 1:3000, *L. kremastos* 1:100, *L. mini* 1:100, *L. autumnalis* 1:3000. The significance of this result is not clear. One other horse, several dogs, and the pigs on the farm were not investigated.

Nine months later, blood was taken from ten of the cows previously tested, of which five showed negligible changes in antibody titre, four had gained antibody, and one remained negative. The antibody titre in the calf had fallen from 1:30,000 to 1:10,000. Of four animals then bled for the first time, one had been in the herd at the time of the outbreak, and had been ill; the other three had been introduced after the outbreak had subsided. All showed titres of 1:1000 or more against *L. pomona*. No sickness had been noticed in the introduced animals, and they may

have been immune when purchased, or have acquired inapparent infections on the farm.

The origin of the outbreak is uncertain. No illness had been noted in the pigs, but the drainage from the pig pen ran over land accessible to the cattle. The farmer had allowed cattle from another herd to graze on his land before the outbreak.

None of the five persons on the farm suffered from clinical leptospirosis at that time, and the owner's serum did not react with *L. pomona*. Nevertheless, as with the cane farm in Table 7, the experience indicates strikingly the weight of infection to which particular groups of rural workers may sometimes be exposed.

The other side of the epidemiological picture is illustrated by the fact that antibodies (alone, or in addition to antibodies against *L. pomona* and the serotypes which cross-react with it) were detected to *H. hyos* in two of the cows, to *L. celledoni* in six, and to the *hebdomadis* serogroup in nine. Two *H. chrysogaster*, 1 *U. caudimaculatus*, 18 *M. lutillus*, 12 *R. assimilis*, 1 *R. rattus*, and 5 *M. musculus* (all included in Table 2) were trapped on the farm in areas to which the cattle had access. Antibodies to *L. hyos* were found in 1 *H. chrysogaster* and 2 *R. assimilis*, 1 of which had leptospires in the kidney, and *L. celledoni* was isolated from 1 *R. assimilis*. No bandicoots were trapped, although the owner of the farm stated that they were numerous. The association between the domestic stock and the small mammals was close, and it seems likely that there had been some exchange of infections between them.

General survey

The results of agglutination–lysis tests on the sera of 253 domestic mammals are set out in Table 9. The discrepancy between the numbers of animals reacting and the numbers with titres to the individual serogroups is accounted for by multiple infections. Except for one dog, cultures were not made from animals in this series.

Cattle. Excluding the 41 dairy cattle discussed above, sera were tested from beef and dairy cattle from widely scattered areas in North Queensland, including other localities in Johnstone shire (16), Atherton Tableland (47), Daintree (35), Charters Towers (24), Ingham (12) and Hughenden (3). Sixty showed titres of 1:100 or more, *hyos* (29), *australis* (16), and *pomona* (12) being the commonest serogroups, although some of the titres to the *australis* serogroup were probably heterologous reactions from *pomona* infections. One animal showed titres of 1:1000 to both *pomona* and *hyos*. Reactions were also obtained with all other serogroups used except *hebdomadis*, which, however, had been recorded in the Palmerston cattle. Twenty-six of 35 sera from twelve Daintree dairy herds contained antibodies at titres of 1:100 or more, including 14 with antibodies to *hyos*, but none to *pomona*.

Pigs. Twenty out of forty domestic pigs and five out of fourteen wild pigs showed agglutinins at titres of 1:100 or more to five serogroups. Pig sera collected at Innisfail from Atherton Tableland animals showed evidence of infections with *L. pomona* and *L. hyos*. The Douglas shire animals (including one wild pig) had antibodies to *icterohaemorrhagiae*, *pyrogenes*, and *australis* serogroups.

Thus, *L. pomona* and *L. hyos* are present in cattle and pigs on the Atherton Tableland and adjoining areas carrying domestic stock. Sutherland *et al.* (1952)

Table 9. *Serological survey of domestic animals*

Species and no. tested	Animals reacting		Numbers reacting with serogroups									
	Numbers	At titres of	*icterohaemorrhagiae*	*canicola*	*pyrogenes*	*australis*	*pomona*	*grippotyphosa*	*hebdomadis*	*hyos*	*celledoni*	*autumnalis*
Cattle, 137	30	1:100	5	1	6	11	2	2	—	13	1	2
	18	1:300	—	—	—	4	3	—	—	12	—	—
	9	1:1000	—	—	—	1	5	—	—	4	—	—
	3	1:3000 or more	1	—	—	—	2	—	—	—	—	—
Pigs, 54	9	1:100	—	—	2	6	2	—	—	—	—	—
	4	1:300	2	—	2	—	—	—	—	—	—	—
	6	1:1000	2	—	—	—	3	—	—	1	—	—
	6	1:3000 or more	—	—	—	2	4	—	—	—	—	—
Sheep, 7	2	1:100	—	—	1	1	1	—	—	—	—	—
	1	1:300	—	—	—	—	1	—	—	—	—	—
Dogs, 34	11	1:100	—	—	3	3	1	—	4	—	—	—
	2	1:300	—	1	—	1	—	—	—	—	—	—
	2	1:1000	—	—	—	1	—	—	—	1	—	—
Cats, 21	—	1:100	—	—	—	—	—	—	—	—	—	—
	1	1:300	—	—	—	—	1	—	—	—	—	—

had reported *pomona* leptospirosis from all dairying areas of the state except the Atherton Tableland. The results suggest also that cattle and pigs may become infected with serotypes belonging to several other serogroups. The animals were not reported as ill, and these serotypes have not been associated with disease in stock in Australia, although antibodies to *icterohaemorrhagiae* and *grippotyphosa* serogroups in both pigs and cattle and to *hebdomadis* serogroup in cattle have been recorded (Wannan, 1955; Forbes, Keast, Wannan & Lawrence, 1955).

Sheep. The wet coastal belt does not carry sheep. The seven sera in Table 9 were collected at an Innisfail slaughter-house from sheep brought from Hughenden in the drier western part of North Queensland. Two reacted with *L. pomona*, at titres of 1:100 and 1:300, and one with *L. zanoni* at 1:100. The significance of these results is uncertain. Leptospiral infections in sheep have not been recorded from Queensland, although they have been reported from other Australian states (Wannan, 1955) and overseas countries (Alston & Broom, 1958).

Dogs. Gray (1940, 1942) has described canine leptospirosis in southern Queensland, but it is seldom recognized by veterinarians in the north. None of the dogs reported here showed signs of sickness, and all but one were contacts of human cases of leptospirosis. One had antibodies to the *canicola* serogroup, but was not associated with a human case. Only three of the fifteen dogs which reacted to a titre of 1:100 or more had antibodies to the serotype that was responsible for the human infection in the household: one of three belonging to a patient with a *medanensis* infection; one at the home of a child with a *zanoni* infection; and one of three at the home of another child with a *zanoni* infection (but one of the other dogs had antibodies at 1:1000 to *L. hyos*). A higher correlation would have been expected, if dogs had been reservoirs, or had formed a significant link in the chain of infection from the small ground mammals to man.

Cats. Cats are common domestic pets in the area. The 21 in Table 9 had been household pets in or near Innisfail, and the sources of the *pomona* infection in one of them can only be guessed. Domestic cats have become feral in the canefields, but this population has not been sampled.

REVIEW OF THE SEROTYPES

It will be convenient, at this point, to review briefly what is known of the status and hosts of the serotypes that occur in North Queensland, as a background to a discussion of their ecology. The findings to date, including, for completeness, those of Battey *et al.* (1964), are epitomized in Table 10.

Leptospira icterohaemorrhagiae

This serotype is present in southern Queensland, but it is not certain that it occurs in the north. Of ten infections in man recorded as *icterohaemorrhagiae* by Derrick (1957), only two were diagnosed from cultures, and absorption tests to exclude other members of the group were not being undertaken at that time. Moreover, the occupational diversity of the cases, including some in cane farmers and timber getters, and the restricted distribution of *R. norvegicus* in the area,

Table 10. *Summary of hosts of leptospiral serotypes in North Queensland*

Serogroups	Serotypes	Relative frequency in man (%)*	Probable maintaining hosts	Probable incidental hosts
Icterohaemorrhagiae	*?icterohaemorrhagiae*	3	?	*?Perameles nasuta*, *?Hydromys chrysogaster*, *?Rattus assimilis*, *?R. rattus*, ?cattle, ?pigs
Canicola	*canicola* *broomi* *bindjei*	4·5	*?Isoodon macrourus* *I. macrourus* *?Melomys* spp.†	*P. nasuta*, *Uromys caudimaculatus*†, *R. rattus*, dogs
Pyrogenes	*zanoni*	26	*R. rattus*, *R. norvegicus*, *Mus musculus*, *?R. s. conatus* (focal)	*P. nasuta*, *I. macrourus*, *H. chrysogaster*, *U. caudimaculatus*, *Melomys lutillus*, *M. cervinipes*†, *R. s. conatus*, *R. assimilis*, dogs
	robinsoni	6	*?U. caudimaculatus*, *?R. s. conatus*	?
Australis	*australis*	29	*R. s. conatus* (in canefields), *R. assimilis* (in rain forest)	*P. nasuta*, *I. macrourus*, *Hypsiprymnodon moschatus*, *H. chrysogaster*, *U. caudimaculatus*, *M. cervinipes*, *M. lutillus*, *R. rattus*, *M. musculus*, *Pteropus conspicillatus*, cattle, pigs, dogs
	bratislava	1	?	?
Pomona	*pomona*	2	Pigs, cattle, *R. assimilis* (in rain forest)	*P. nasuta*, *I. macrourus*, *U. caudimaculatus*†, *M. cervinipes*, *R. s. conatus*†, *R. rattus*, dogs, cats
Grippotyphosa	*grippotyphosa*	0·3	*R. s. conatus*†	*Thylogale stigmatica*, *P. nasuta*†, *I. macrourus*†, *U. caudimaculatus*†, *R. rattus*
Hebdomadis	*kremastos* *mini* *medanensis*	12 3 1	*P. nasuta*, *I. macrourus* *I. macrourus* *P. nasuta*†, *I. macrourus*†	*Trichosurus vulpecula*, *R. assimilis*, cattle, dogs
Hyos	*hyos*	6	Cattle, pigs, *?R. assimilis* (in rain forest)	*P. nasuta*, *I. macrourus*†, *H. chrysogaster*, *U. caudimaculatus*, dogs
Celledoni	*celledoni*	6	*?P. nasuta*, *?I. macrourus*, *?M. cervinipes*, *?R. assimilis*	*H. chrysogaster*†, *M. lutillus*, cattle

* From Derrick's (1957) analysis of 382 cases.
† From Battey *et al.* (1964).

indicate that the local epidemiology differs considerably from that of classical Weil's disease.

L. canicola

Only two infections in man have been identified since the serotype was reported from Queensland by Sinnamon *et al.* (1953); both were in the Mulgrave shire. There is no evident association with dogs, and the maintaining hosts are either bandicoots or not among the species investigated.

L. broomi

This has been the most frequent serotype in the *canicola* serogroup infections of man in the area, and it has been isolated also from *I. macrourus* and *R. rattus*. The high incidence of serogroup antibodies in the bandicoot would suggest that it is the maintaining host. However, the *canicola* serogroup cases studied by Derrick (1956) showed a significant preponderance ($P < 0{\cdot}05$) away from canefield occupations, so other reservoirs may also be involved.

L. bindjei

The occurrence of this serotype was not recognized before our work was completed, but it is now known to have produced several human infections in the Douglas shire, including one on the cane farm where it was isolated from *M. lutillus*. It has since been recovered also from *M. cervinipes* in the same district (Battey *et al.* 1964).

L. zanoni

Human infections with this common serotype were widespread in the area, with a significant predominance over other serotypes in the Johnstone shire, and an almost equal incidence in and away from canefields; it also caused urban leptospirosis in Innisfail. The introduced rodents are the maintaining hosts, but it has been recorded from many other species, although it appears to intrude but little into the rain forest.

L. robinsoni

Infections in man have been limited almost entirely to the southern half of the area, and were equally distributed in and away from canefields. It has been isolated from *U. caudimaculatus* and *R. s. conatus*, and may have contributed to the '*pyrogenes*' antibodies found in other species.

L. australis

This serotype is the cause of classical canefield fever in North Queensland. It is widespread, with a significantly high incidence in the Mulgrave 3 division, and a highly significant association both with canefields and with *R. s. conatus*. It is highly pathogenic to laboratory mice (Emanuel, 1959); but field experience has shown that it can produce benign infections in a variety of hosts, and *R. assimilis* appears to be able to maintain foci of infection in rain forest.

L. bratislava

This serotype was isolated from a cane-cutter and from cane trash at Babinda. It was named *L. esposito* by Smith & Brown (1955), but was subsequently found by Wolff & Bohlander (1961) to be serologically identical with *L. bratislava*. Owing to its cross-agglutination pattern, it can be identified only when isolated in culture, and its animal hosts are still unknown.

L. pomona

Pigs appear to be the primary hosts in southern Queensland, with a considerable overflow into cattle, in which it may cause serious clinical disease, and from both pigs and cattle into people who have contact with them. The same sequence occurs in North Queensland, but there is an appreciable amount of infection in the small ground mammals as well. A variant has been found in *R. assimilis*, and it is not yet clear how far it may contribute to infections in other species, including man.

L. grippotyphosa

This serotype was isolated from a cane-cutter in the Douglas shire (Smith & Brown, 1955), and a second infection has recently been identified serologically in a soil chemist who was working in Brisbane with samples of soil from the same district (Tonge & Smith, 1961). Antibodies were found in 3 *R. rattus* from the same area as the human cases and in one *Thylogale stigmatica* from near Innisfail, but it now seems probable that *R. s. conatus* is the maintaining host in the Douglas shire (Battey *et al.* 1964).

L. kremastos

Originally isolated from a dental nurse whose home was on a cane farm near Babinda, *L. kremastos* was subsequently found to be more prevalent in human infections in the Johnstone shire, and to be almost equally distributed in and away from canefields. The reservoirs are marsupials, and it has been isolated from both species of bandicoot.

L. mini

In contrast with *L. kremastos*, *L. mini* was first isolated from an Innisfail hospital worker, and then found to be more prevalent in Mulgrave 3 and significantly associated with canefields. It has been isolated from *I. macrourus*, but it is not known whether it may occur in other species.

L. medanensis

This serotype has been recorded so far only from five patients in the Douglas shire. It was not recovered from animals, and significant titres to it were not recorded from local bandicoots, but it has subsequently been isolated from both species (Battey *et al.* 1964).

Of the infections identified only as *hebdomadis* serogroup, the habitats of the animals suggest that those in *R. assimilis* in rain forest and in the Palmerston dairy

cattle were more likely to have been caused by *L. kremastos* than *L. mini*, but those in dogs on the more southern cane farms could have been due equally to either. The serum of the possum (*T. vulpecula*) from the Atherton Tableland (and of an unidentified marsupial from the same area, not included in Table 2) gave a significant titre only to *L. medanensis*, as did that of a dog belonging to a patient with *medanensis* leptospirosis.

L. hyos

The epidemiology of *hyos* infection in man is similar to that of *L. pomona*, occupational contact with cattle and pigs generally being an essential feature. However, some of the northern cases were in forest workers. There was no clear indication of infection in the thirteen wild pigs examined, but an appreciable incidence in *P. nasuta*, *U. caudimaculatus*, *R. assimilis*, and the water rat, *H. chrysogaster*. There is reason to believe that this serotype, like the variant of *L. pomona*, can be maintained in rain-forest mammals not in contact with cattle, and it may be significant that the strains isolated from these animals show distinguishing subserotype characteristics.

L. celledoni

This distinctive serotype was described by Smith *et al.* (1954), and it proved to be widespread in the area, twenty-four human infections being recorded, including the only case of urban leptospirosis in Cairns. It seems to be associated primarily with forest animals, but it has also been found in *I. macrourus* and *M. lutillus* in canefields, and it may infect cattle.

DISCUSSION

The concept of principal and incidental hosts (at least so far as man is concerned) has been inherent in the definition of zoonoses, but Audy (1958) gave it precision by introducing the term 'maintaining hosts', and drawing a clear distinction between maintaining and incidental hosts in the animal populations. We have inferred that there are particular species of mammals on which the populations of the leptospiral serotypes in North Queensland depend for survival, and the most important questions to answer, from both practical and theoretical points of view, are whether this is true, and, if it is, how these maintenance associations are to be recognized, and what conditions influence their stability and effectiveness.

It is a basic principle in any study of the interactions between hosts and parasites that no two host–parasite associations are precisely alike. Whether they differ so little as not to affect a particular inquiry, or so much as to be fundamental to it, can be determined only by observation. It follows that we should examine each of the associations separately, and it is a defect in the work reported here—as it is also in the Malayan studies of Gordon Smith *et al.* (1961*a*, *b*)—that that cannot be done with any real precision. The species of hosts can be stated precisely, but not those of the leptospires. There are two reasons for this. One is that, although the serotype is treated as a species for purposes of nomenclature, many serotypes have not yet been shown to be stable genetic entities. The other is that, as it is impractic-

able to identify serological reactions to infection beyond serogroup, the identity of an infecting serotype can often only be guessed. This introduces an unfortunate element of vagueness into a situation that is already inherently complex by reason of the numbers of serotypes and host species involved. These weaknesses must limit the precision, though not necessarily the nature, of the conclusions that can be drawn.

Gordon Smith *et al.* (1961*a*) defined the requirements for survival of populations of leptospires, and we may present them in a somewhat different way here, in order to define the ecological problems more clearly. They are:

(1) A host–parasite interaction that provides opportunities for spread of leptospires in the host population without significantly reducing its level and activity.

(2) An independent set of favourable properties of the hosts, including habits, habitats, longevities, ranges, and population densities.

(3) Another independent set of external factors favouring survival (and perhaps multiplication) of leptospires outside the bodies of the hosts.

We are concerned, in this paper, primarily with the first.

Babudieri (1958) has given a clear statement of the basic relationship between host and parasite that characterizes a maintenance association. After describing the course of an infection, from early leptospiraemia to later lodgement in the kidneys, and, *in some infections only*, development of dense colonies of leptospires in the convoluted tubules whence the organisms are shed into the urine, he goes on: '. . . a state of biological equilibrium is easily established between some serotypes of leptospires and some animal species. This equilibrium is either lacking or much more difficult to establish in other cases. Both components of this symbiotic association play a part in this phenomenon. In fact, it is observed that, while certain animals easily become carriers of some serotypes of *Leptospira*, they do not become so for others. This confirms anew the biological validity, discussed by some investigators, of the subdivision of *Leptospira* into serotypes; at the same time it demonstrates the fact that the distinction made initially between temporary shedders and persistent carriers, although unconventional, is one that rests on a solid if poorly explicable biological basis. Any animal susceptible to infection by leptospires may become a temporary shedder, but only those animal species that present a particular condition of biological sympathy for a determined serotype of *Leptospira* can become carriers.'

Our approach has been somewhat different, and it provides a link between Babudieri's account of what happens in the host and what may happen in the field. Emanuel (1959) inoculated laboratory mice with blood-clot from patients with acute leptospirosis, and found that the resulting infections formed a series which could be divided conveniently into three grades:

I. Severe, with obvious illness and high mortality, the survivors becoming chronic urinary carriers (*L. australis*, *L. grippotyphosa*).

II. Moderate to mild, with few clinical signs, usually ending in recovery and continued urinary excretion of leptospires (*L. icterohaemorrhagiae*, *L. broomi* (recorded as '*canicola*'), *L. zanoni*, *L. robinsoni*, *L. bratislava*, *L. hyos*).

III. Inapparent, with no clinical signs, often transient, but mice sometimes

becoming urinary carriers (*L. pomona*, *L. kremastos*, *L. mini*, *L. medanensis*, *L. celledoni*).

There was some difference between strains, but the general pattern was reasonably constant, and the following strains subsequently recovered from animals have been found to conform to it: 25 *L. australis*, 8 *L. zanoni*, 1 *L. robinsoni*, 2 *L. hyos*, 11 *L. ?pomona*, 1 *hebdomadis* serogroup and 2 *L. celledoni*, while 1 *L. bindjei* produced similar grade II infections to *L. broomi*.

We may, then, visualize the mouse as a 'good' host for the serotypes in grade II. Those in grade I might survive as local populations (but probably with marked epizootic fluctuations if they did), while those in grade III, although not harming the host, are unlikely to reach the exterior in sufficient numbers to ensure maintenance of the population.

It does not follow that infections by these serotypes would produce the same pattern of response in other species of mammals. Indeed, as Babudieri showed in his review, there is evidence that they do not. Man is a case in point, human infections in Queensland differing both in gradation of severity and types of reaction from those in the mouse. In the animals, *L. zanoni* produces moderate to mild infections in mice, but three strains from patients all produced severe reactions in guinea-pigs. A culture of *L. zanoni* obtained from a wild *M. musculus* was inoculated into four laboratory mice, one *Melomys lutillus*, one *M. cervinipes* and one *I. macrourus*. It produced inapparent infections in all, but urinary carriers only in the mice. On the other hand, *L. bindjei* produced urinary carriers in 2 *M. cervinipes*, 1 *M. lutillus*, and at least six of the eight laboratory mice into which it was inoculated. Similar results have been obtained in other parts of the world. Thus, Walch-Sorgdrager (1939) showed experimentally that *L. icterohaemorrhagiae* regularly produced persistent urinary infections in *R. norvegicus*, whereas *L. canicola* produced only transitory infections, with rare, brief excretion of leptospires. Conversely, Packchanian (1940) demonstrated that different species of rodents differed markedly in their susceptibility to experimental infection with *L. icterohaemorrhagiae*, while Stavitsky & Green (1945) and Neghme, Christen, Jarpa & Agosin (1951) found that different races of a host species differed in their susceptibility to infection with a single strain of *Leptospira*.

We would, then, expect the situation in the field to present a mosaic of host–parasite relationships, with the order of the serotypes in the graded sequences differing from species to species of the host range.

It would have been desirable to test this hypothesis by experimental infections in the wild species, but that has not been possible for two reasons. One is that most of the serotypes studied lose their adaptations to parasitic life so quickly when passaged in culture that it is necessary to work directly from the original hosts, which means blind inoculation and a continuous supply of experimental hosts. The other is that adequate numbers of native animals reared in the laboratory have not been available. It has, consequently, been necessary to seek for an indirect method of evaluation, and we have placed most reliance on the excretion index for this purpose, because it would be less influenced by external variables than the overall infection or excretion rates.

Sick animals are not likely to enter traps, so the method of collecting would be biased against detecting grade I infections. Survivors would have a high excretion index, but the overall infection rate would be low, unless there were an epizootic, and no indication of one has been seen, either in the general or mark–recapture observations. Moreover, although no deductions can be made about young animals, comparison of the grown animals in the mark–recapture experiments has revealed no regular loss of weight or condition (Harrison & Emanuel, 1960) and no reduction in the monthly survival rate (Emanuel & Harrison, 1961) of those with infections as compared with the uninfected animals in the populations.

On the other hand, a combination of high index with high rate may, with one exception to be noted below, be accepted as good evidence of a grade II infection. More simply, when an abundant species of animal has a notably higher excretion rate of a particular serotype than any other species, then it can usually be accepted as a maintaining host of that serotype in that area. Thus, *R. s. conatus* was clearly a maintaining host of *L. australis* in most canefields sampled, *R. norvegicus* of *L. zanoni* in Innisfail town, *M. musculus* of *L. zanoni* in the cane farm in Table 7, and *P. nasuta* of a member or members of the *hebdomadis* serogroup (Table 4). A high index with a low rate is more difficult to assess, because it might be an expression, either of survival from severe infections, or of grade II under conditions of reduced frequency of transmission. The latter is more likely, particularly when the combination is observed in different samples or over a period, and we would therefore add *L. zanoni* in *R. rattus*, *L. ?pomona* in *R. assimilis*, and possibly *L. hyos* in *R. assimilis* to the associations that are accepted as maintaining ones.

Grade III infections would be characterized in the field by low excretion indices accompanying infection rates which would vary according to the amount of overflow from the maintaining hosts. There are numerous examples of this sort of relationship in Table 4. There is one condition under which the excretion index would rise. Some animals with this grade of infection do excrete leptospires for a period. When there are dense populations of maintaining and incidental hosts at a time when the environment is particularly favourable for transmission, the proportion of recent infections in the sample would rise, and the numbers excreting leptospires would be expected to rise with it. Some of the *L. zanoni* infections in *R. s. conatus* and of *L. australis* in *R. rattus*, as well as of both serotypes in the bandicoots, may belong to this category, which it should be possible to recognize from collateral information collected with the sample. The epidemiological significance of incidental hosts has been discussed by Audy (1958), and we have no specific information to add from our own experience.

Gordon Smith *et al.* (1961*a*) adopted a similar ratio to assess the infections they studied, although they seemed to consider that it depended on characteristics of the host species, rather than of particular host–parasite associations, and they added the caution that it could be applied only when infection leaves recognizable serological evidence that it has occurred. They also considered that their serotypes could be arranged in a graded series of antigenic capacity in rats (apparently independently of species), from *L. icterohaemorrhagiae* at the top to *L. hyos*, which

produced no antibodies at the 1:200 level, at the bottom. We have analysed our records to test both possibilities, with the following results:

(1) Except for the *R. s. conatus–L. australis* association, the bandicoots produced antibodies at consistently higher titres than the rodents against three serogroups (*pyrogenes*, *australis*, *pomona*) for which comparison was possible, and equally high titres against the *canicola* and *hebdomadis* serogroups which they maintained.

(2) Titres to *L. australis* in *R. s. conatus* were higher than in any other association in rodents, including *L. australis* in other species.

(3) Titres to *L. zanoni* were higher in the introduced rodents than in the native species.

(4) *L. australis* gave a higher incidence of antibodies than *L. zanoni* in excreting rats, and consistently higher titres than *L. zanoni* in bandicoots, maintaining rodent hosts, and incidental rodent hosts.

(5) Of three bandicoots and sixteen rodents infected with *L. hyos* (including those in the mark–recapture experiments), five rodents were excreting, and four of these had antibody titres less than 1:100; in the whole series, nine had titres of 1:100, three of 1:300, and four of 1:1000 or more. *L. hyos* thus appears to stimulate antibody production less readily than other local serotypes (though better than the Malayan strain), but the differences were not statistically significant.

These findings do not invalidate use of the excretion index for comparison of the North Queensland records. They show, too, that both suggestions of the Malayan workers are partly correct for our data, though the differences recorded are probably no more than expressions of the varied specific adaptations between hosts and parasites that Babudieri (1958) and we have described.

The index might also be affected by independent variations in the duration of excretion and persistence of antibodies. Both were followed for considerable periods in some of the mark–recapture studies (Harrison & Emanuel, 1960), and some variations were observed, but they were not of a magnitude that would materially reduce the statistical usefulness of the index.

Three aspects of the problem remain to be considered. The first is the excretion index that would mark a limit below which an independent association could not be maintained. It is likely to be quite variable, depending on the sum of the ecological factors enumerated earlier, and it is not to be forgotten, too, that quite a number of parasites seem to be able to maintain an extraordinarily tenuous existence under circumstances that are, at present, a complete mystery. We have, very tentatively, selected an index of 0·05 (equivalent to 5% of infected animals becoming chronic urinary carriers) as about the lowest at which an association is *likely* to be a maintaining one under favourable conditions, and 0·10 as a possible limit in less favourable conditions. These levels would fit the suspected maintenance of *L. mini* by *I. macrourus*, but more data are needed to define them with any confidence.

The second point is what happens when two or more grade II hosts for a given serotype occur together. There is no reason why it should not happen in nature, and it seems likely that some of the situations studied by the Malayan workers, as

well as our own experience with *L. celledoni* in rain-forest animals, may be examples of it. Presumably, the collective population of hosts serves as a unit, their combined status being the resultant of their relative numbers, positions in the grade, and habits in the field. It might be difficult to distinguish this situation from one in which there is an unusually high excretion index in incidental hosts, so it would be essential to test the findings in different localities and at different times before drawing conclusions from them.

The third point is that situations that are stable in one country may have little resemblance to equally stable situations in another. Racial differences between leptospires and between their mammalian hosts have already been mentioned, the dynamics of the associations undoubtedly differ, too, and there are many examples of a serotype being supported by one host in one country and a different one in another, *L. bataviae* by *R. rattus diardi* in Indonesia and *R. argentiventer* in Malaya and the *hebdomadis* serogroup by rodents in Malaya and marsupials in Australia being two striking examples. Some differences may be more local, and we still lack an explanation of the apparent absence of infection with *L. zanoni* in a sample of 129 *R. rattus* from the Douglas and Mulgrave shires.

To sum up, the concept of a mosaic of host–parasite relationships, each parasite having its own limited number of potential maintaining hosts and a larger, more widely shared range of incidental hosts, is well supported by our data. We believe it to be fundamental in understanding the ecology of the leptospiral infections, because it provides an element of relative stability on which the more variable factors may act. It incidentally provides support for the view that the leptospires are primarily parasites, because it is difficult to imagine how organisms that were primarily saprophytic could have evolved such patterns.

A mosaic of ecological factors is imposed on the host–parasite mosaic, resulting in a combined mosaic of foci of infection, large and small, and producing the varied associations that we see in the field. Focality, indeed, has been a striking finding of all the epidemiological inquiries that have been made in North Queensland, and the mark–recapture studies were designed to elucidate the variables that produce it. The results obtained from them have been summarized by Harrison & Emanuel (1962), and will be presented in greater detail in another paper. It may then become possible to examine the problem of controlling the incidence of infection in man.

SUMMARY

Leptospirosis is a zoonosis that causes appreciable ill health and economic loss in North Queensland. Fourteen serotypes of *Leptospira* were known to infect man in the area, and information on their local distribution and seasonal and occupational incidence had been obtained. The next step in the investigation was to determine the reservoir hosts and estimate their significance.

Wild animals examined comprised 5 monotremes, 643 marsupials, 2355 rodents, 67 bats, 30 birds, 28 reptiles, and 21 toads. Evidence of leptospiral infection was obtained from 223 marsupials, 309 rodents, and 6 fruit-bats. Analysis indicated that the principal maintaining hosts were:

Peremeles nasuta—of *L. kremastos* (and possibly *L. mini*) in canefields and rain forest.

Isoodon macrourus—of *L. broomi*, *L. mini*, and *L. kremastos* in canefields.

Rattus rattus, *R. norvegicus* (focal), and *Mus musculus* (focal)—of *L. zanoni* in town and canefields.

Rattus sordidus conatus—of *L. australis* in canefields.

Rattus assimilis—of foci of *L. australis*, a variant of *L. pomona*, and probably of *L. hyos* in rain forests.

Random infections were also encountered, but the maintaining hosts of the other serotypes known to infect man in the area (*icterohaemorrhagiae*, *canicola*, *bindjei*, *robinsoni*, *bratislava*, *grippotyphosa*, *medanensis*, *celledoni*) were not determined.

Domestic animals examined included 137 cattle, 54 pigs, 7 sheep, 34 dogs, 31 cats, and a dairy herd in which there was an epizootic of *pomona* leptospirosis. Again, there was a wide scatter of infection, but, apart from the long-known association of *L. pomona* and *L. hyos* with cattle and pigs, there was no evidence of maintaining hosts in this series.

As indicated in the Introduction, the work reported here was part of a planned investigation into the epidemiology of leptospirosis. Some of the early material was collected by Dr M. J. Mackerras, Dr R. L. Doherty, Mrs D. G. Delamoir, and Miss C. J. Ross, who had previously been stationed at the Institute's Field Station, and we are indebted to Mr D. W. Lavers, Mr S. G. Knott, and Mr R. E. Dunham, of the Queensland Department of Agriculture and Stock, for a number of sera from domestic animals. Many of the mammals were identified by Dr W. A. McDougall, of the Queensland Department of Agriculture and Stock, Mr E. le G. Troughton, then of the Australian Museum, Sydney, Mr B. J. Marlow, then of the C.S.I.R.O. Wildlife Section, Canberra, and by Dr J. L. Harrison, Dr M. J. Mackerras, and Mr R. Domrow of the Institute. We are indebted also to Mrs M. Macgregor, Librarian of the Institute, for considerable help with the literature.

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J. Hyg., Camb. (1964), **62**, 485
Printed in Great Britain

The epidemiology of leptospirosis in North Queensland

II. Further observations on the hosts in the Mossman district

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(*Received* 1 *June* 1964)

The studies of leptospiral infections in North Queensland recorded in Part I of this series (Emanuel, Mackerras & Smith, 1964) were undertaken mostly in the Johnstone and Mulgrave shires, and they consequently included little information about two serotypes which had been isolated only from human patients in the Mossman district (16° 26′ S., 145° 22′ E.) of the Douglas shire farther to the north. Serotype *medanensis* was recovered from two brothers (Ives), canefarmers, in 1951 (Smith *et al.* 1954), and *grippotyphosa* from a cane-cutter (Valbuzzi) in 1954 (Smith & Brown, 1955). The survey reported here was made in July and August, 1962, primarily to obtain information on the animal hosts of these two serotypes.

MATERIALS AND METHODS

The topography of the district has been described in Part I, and details of the areas trapped are shown in Fig. 1. All were within 10 miles of the town of Mossman.

Animals were trapped by the methods described by Harrison (1962); 2750 trap-nights produced 221 animals, of which 216 were in a state suitable for examination. A further 30 animals were captured by schoolchildren and are listed under Miscellaneous in Table 1. Some of the areas were selected with reference to known cases of leptospirosis, e.g. the farms where Ives and Valbuzzi worked, and Schild's farm that was the source of the soil which probably infected a Brisbane scientist with *grippotyphosa* (Tonge & Smith, 1961). Others were chosen with a view to their suitability for certain scrub typhus investigations that were performed concurrently, and are listed under names of occupiers of farms.

Two hundred and forty-six animals were examined, comprising 85 bandicoots of two species and 161 rodents of seven species. Their distribution in relation to species and habitats is set out in Table 1. Although the number of such species was small, the habitats observed were similar to those described by Harrison (1962), with two exceptions. Four *I. macrourus* and 6 *M. lutillus* were caught in rain-forest. However, the various types of rain-forest encompassed here were small pockets in or adjacent to sugar cane and grassland. As the survey was conducted at the height of the cane burning and cutting season, disturbance and movement of

species could be expected. No animal trapped at Dayman Point, forest and grassland, showed evidence of infection with leptospires.

At a temporary field laboratory established at the Mossman District Hospital, the traps were removed from the cloth bag and suspended over clean white enamel trays for the collection of urine. The traps were kept in a place that was under

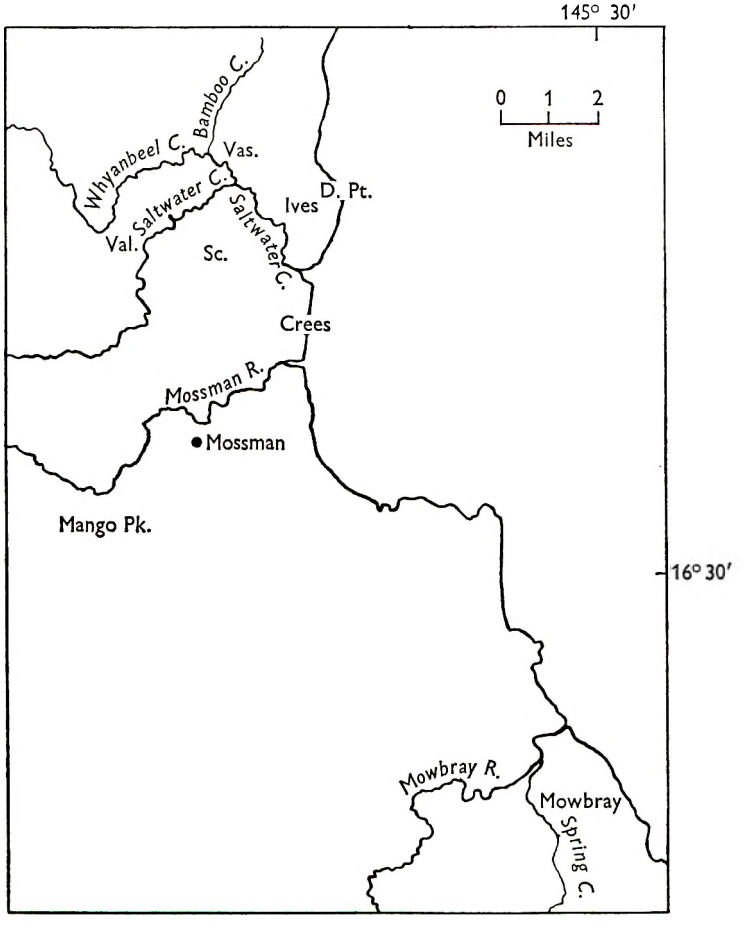

Fig. 1. Map of the Mossman district, showing trapping areas: Vas., Vassalini; Val., Valbuzzi; Ives; D. Pt., Dayman Point; Sc., Schilds; Crees; Mango Park; Mowbray.

constant observation and microscopic examination was carried out within a few minutes of the passing of urine. Most rodents would pass a few drops of urine if excited by blowing in the face, but the marsupial bandicoots seldom provided specimens. Urine was collected from the bladder of these as soon as the sterile dissection was completed. One or two drops of urine were placed on a clean, glass slide, covered with a 22 mm. square coverslip and examined for leptospires using dark ground illumination and a magnification of approximately 400. The presence of leptospires was recorded and graded from + for only an occasional organism to + + + + for a heavy infection.

The animals were transferred to a metal box with glass lid and killed with an

Table 1. *Distribution of animal species in relation to trapping areas and habitat*

Species	Area and habitat															
	Vassalini		Mowbray		Mango Park		Dayman Point	Crees	Schilds		Ives		Valbuzzi		Miscellaneous	Total
	Rain forest	Secondary woodland and grassland	Rain forest	Secondary woodland and grassland	Rain forest	Secondary woodland and grassland	Secondary woodland and grassland	Farm	Rain forest	Farm	Secondary woodland and grassland	Farm	Rain forest	Farm	Farm and grassland	
Perameles nasuta	4	4	—	—	—	—	7	—	—	—	—	—	—	—	—	15
Isoodon macrourus	1	—	—	1	2	2	—	11	1	3	—	2	1	18	28	70
Hydromys chrysogaster	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
Uromys caudimaculatus	5	—	—	—	1	—	—	—	—	—	—	—	1	—	—	7
Melomys cervinipes	10	10	3	5	—	—	1	—	—	2	—	3	—	2	—	36
M. lutillus	1	6	5	7	3	2	12	2	1	4	1	16	—	16	—	76
M. spp. (young)	—	—	—	—	—	—	—	—	—	—	—	—	1	1	—	2
Rattus rattus	—	—	—	—	1	—	2	—	—	—	—	—	—	—	1	4
R. assimilis	—	1	—	—	—	1	1	—	—	—	—	—	—	—	—	3
R. sordidus conatus	—	—	—	—	1	6	—	3	—	—	2	6	—	13	—	31
Total	22	21	8	13	8	11	23	16	2	9	3	27	3	50	30	246

ether-soaked swab of cotton-wool and gauze. Immediately after death sterile dissection was carried out and media inoculated with a very small piece of kidney cortex. Three $\frac{1}{4}$ oz. McCartney bottles, each containing 3 ml. of media, were inoculated from each animal. The media used were:

(*a*) 'TPB', Tryptose phosphate broth (0·2 %) plus rabbit serum (10 %).

(*b*) 'TPA$_1$', Tryptose phosphate broth (0·2 %) and agar (0·15 %) plus rabbit serum (10 %).

(*c*) 'TPA$_2$', Tryptose phosphate broth (0·2 %) and agar (0·15 %) plus rabbit serum (20 %).

A haemoglobin supplement and cyclohexamide were added to each medium.

Cultures were incubated at 30° C. and examined microscopically with dark-ground illumination on the 7th, 14th, 21st and 28th days. If there was no growth, they were then discarded. Positive cultures were subcultured and forwarded by air to the Laboratory of Microbiology and Pathology, Brisbane, for identification.

Table 2. *Comparison of survey methods in bandicoots and rodents examined by all three methods*

Species	No. examined	No. of infections determined by Culture	Dark-ground examination	Serology	All methods
P. nasuta	15	2	1	4	5
I. macrourus	59	4	4	24	25
H. chrysogaster	2	1	0	2	2
U. caudimaculatus	7	4	1	5	5
M. cervinipes	36	3	1	5	6
M. lutillus	57	0	0	0	0
R. rattus	4	0	0	0	0
R. assimilis	3	0	0	0	0
R. s. conatus	30	7	8	8	9
Total	213	21 (10 %)	15 (7 %)	48 (23 %)	52 (24 %)

Leptospires were isolated from twenty-one animals, but as five of these had both kidneys cultured, there were twenty-six positive kidneys. The results of the three media are:

leptospires were grown from 17 kidneys in 'TPB',

leptospires were grown from 18 kidneys in 'TPA$_1$',

leptospires were grown from 19 kidneys in 'TPA$_2$',

this suggests that no medium is better than the others, but that the more media inoculated, the greater the chance of growing leptospires.

Heart blood was collected, the serum separated and forwarded to the Laboratory of Microbiology and Pathology for serological investigation. The sera were screened by the microscopic agglutination test against the following serotypes: *ictero-haemorrhagiae*, *canicola*, *broomi*, *zanoni*, *robinsoni*, *australis*, *bratislava*, *pomona*, *grippotyphosa*, *medanensis*, *kremastos*, *mini*, *hyos*, *celledoni* and *autumnalis*. Serological results were interpreted with the conservatism described in Part I.

Table 2 records the number of infections determined by culture, dark-ground

examination and serology in 213 animals in which all three procedures were used. The percentage of infections, proved by culture, in this series was double that of Part I. However, the ratio of culture to dark-ground examination was the same for both series, 2:1. This suggests that the higher culture rate was probably due to a focus of infection in the district rather than improved culture media.

Omitted from this series is a group of 20 *M. lutillus* and *Melomys* (young) in which organisms resembling leptospires were seen in the urine but not grown by kidney culture. Their sera did not show any leptospiral antibodies even when tested with an extended range of thirty-two serotypes representing eighteen groups. As tissues had been preserved in formalin for other purposes, sections of two kidneys from animals with a + + + and + + + + excretion in their urines were cut and stained but no leptospires were seen. It is probable that the organisms seen in their urine were not leptospires; they are certainly not comparable with the '*pomona*-like' strains mentioned in Part I.

RESULTS

Cultures and sera

Twenty-two leptospiral strains were cultured from 21 of 246 animals tested (Table 2). Strains of two serotypes, *medanensis* and *celledoni*, were grown from the kidney of one short-nosed bandicoot, *I. macrourus*, which also showed serological evidence of infection with *grippotyphosa* and *hyos*. The strains were distributed amongst seven serotypes, namely *grippotyphosa* (8), *medanensis* (5), *zanoni* (4), *hyos* (2), *celledoni* (1), *australis* (1) and *bindjei* (1).

Sera from 233 animals were tested. Forty-eight, twenty-eight bandicoots and twenty rodents, showed the presence of antibodies to a titre of 1:100 or more (Table 2). Thirty-six of these exhibited a serological pattern characteristic of infection with organisms of a single serogroup and twelve showed evidence of multiple infection.

Seven of the 8 *grippotyphosa* strains were isolated from *R. s. conatus*, the cane-field rat. Six of these strains were isolated from animals trapped in the Valbuzzi area and one trapped in the Ives area. The eighth *grippotyphosa* strain was cultured from an *I. macrourus* trapped in the Valbuzzi area. All the *grippotyphosa* strains were grown from animals caught in sugar cane. Ten animals, in addition to those from which *grippotyphosa* isolates were obtained, showed serological evidence of *grippotyphosa* infection. Seven of these, all *I. macrourus*, were trapped in sugar cane; 2, a *P. nasuta* and a *U. caudimaculatus*, were caught in rain forest; the 10th was an *I. macrourus* trapped on the pineapple farm (Schilds) that provided the soil which probably infected the Brisbane scientist quoted above. Thirteen of the animals showing evidence of *grippotyphosa* infection came from the same area as the patient Valbuzzi, from whom *grippotyphosa* was first isolated in Australia. The titres ranged from 1:100 to 1:30,000. Three of the animals from which *grippotyphosa* was isolated had antibody titres of only 1:100.

Two of the five *medanensis* strains were isolated from the long-nosed bandicoot, *P. nasuta*. Both of these were trapped in the Vassalini area. The remaining three

Table 3. *Occurrence of infections in animals as determined by culture and serology*

Species	No. examined	Isolations: *bindjei*	*zanoni*	*australis*	*grippotyphosa*	*medanensis*	*hyos*	*celledoni*	Total	Antibodies: *canicola* group	*pyrogenes* group	*australis* group	*pomona*	*grippotyphosa*	*hebdomadis* group	*hyos*	*celledoni*	Total
P. nasuta	15	—	—	—	—	2	—	—	2	2	—	—	—	1	2	1	1	7
I. macrourus	59	—	—	—	1	3*	—	1*	4	2	4	1	8	9	5	1	1	31
H. chrysogaster	2	—	—	—	—	—	1	—	1	—	1	—	—	—	—	1	1	3
U. caudimaculatus	7	—	2	1	—	—	1	—	4	2	2	1	3	1	—	1	—	10
M. cervinipes	36	1	2	—	—	—	—	—	3	1	2	—	2	—	—	—	—	5
M. lutillus	75	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	0
M. spp. (young)	2	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	0
R. rattus	4	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	0
R. assimilis	3	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	0
R. s. conatus	30	—	—	—	7	—	—	—	7	—	1	—	1	7	—	—	—	9
Total	233	1	4	1	8	5	2	1	22/21	7	10	2	14	18	7	4	3	65

* Double infection.

Table 4. *Infections in animals from different habitats*

Species	Rain forest: No. examined	*canicola* group	*pyrogenes* group	*australis* group	*pomona*	*grippotyphosa*	*hebdomadis* group	*hyos*	*celledoni*	Secondary woodland and grassland: No. examined	*canicola* group	*pyrogenes* group	*pomona*	*hebdomadis* group	Cultivation: No. examined	*canicola* group	*pyrogenes* group	*australis* group	*pomona*	*grippotyphosa*	*hebdomadis* group	*hyos*	*celledoni*
P. nasuta	4	—	—	—	—	1	1	1	1	11	2	—	—	1	0	—	—	—	—	—	—	—	—
I. macrourus	4	1	—	—	1	—	1	—	—	4	—	1	2	—	62	1	3	1	5	9	4	1	1
H. chrysogaster	1	—	1	—	—	—	—	—	—	0	—	—	—	—	1	—	—	—	—	—	—	1	1
U. caudimaculatus	7	2	2	1	3	1	—	1	—	0	—	—	—	—	0	—	—	—	—	—	—	—	—
M. cervinipes	10	1	—	—	2	—	—	—	—	19	—	1	—	—	7	—	1	—	—	—	—	—	—
M. lutillus	6	—	—	—	—	—	—	—	—	32	—	—	—	—	38	—	—	—	—	—	—	—	—
M. spp. (young)	0	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	—	—
R. rattus	1	—	—	—	—	—	—	—	—	2	—	—	—	—	1	—	—	—	—	—	—	—	—
R. assimilis	0	—	—	—	—	—	—	—	—	3	—	—	—	—	0	—	—	—	—	—	—	—	—
R. s. conatus	1	—	—	—	1	—	—	—	—	8	—	—	—	—	22	—	1	—	—	7	—	—	—

strains of *medanensis* were grown from *I. macrourus* caught in the Valbuzzi area. These sera reacted with all three serotypes of the *hebdomadis* group, but their *medanensis* titres were at least ten times greater than *kremastos* and *mini* titres. Two other animals showed serological evidence of infection with the *hebdomadis* group; an *I. macrourus* trapped at Vassalini reacted only with *medanensis*; and an *I. macrourus* caught on a farm by a schoolchild showed higher titres to *kremastos* and *mini* than to *medanensis*. The type of country varied from rain forest to grassland and sugar cane.

A strain of *bindjei* was grown from a *M. cervinipes* trapped in rain forest in the Vassalini area. Antibodies to the serotypes in the *canicola* group were found in six other sera from the species *P. nasuta* (2), *I. macrourus* (2) and *U. caudimaculatus* (2).

Strains belonging to serotypes *zanoni*, *hyos* and *celledoni* were also isolated (Table 3). Antibodies to serotype *pomona* were demonstrated in the sera of 14 animals. Six of these were *I. macrourus* trapped by schoolchildren on various farms in the district. The others were *I. macrourus* (2), *U. caudimaculatus* (3), *R. s. conatus* (1), *M. cervinipes* (2) which were trapped in rain forest and grassland. No strains of *pomona* were isolated. A similar finding in relation to apparent *pomona* infections in *R. assimilis* was reported in Part I.

The association of infected animals and habitats are set out in Table 4. It is interesting that *M. lutillus* (76 trapped) showed no evidence of infection.

Multiple infections

A high incidence of multiple infections was noted in both rodents (30%) and bandicoots (21%). This differs from the results of the earlier survey in which antibody patterns in rodents indicated relatively few multiple infections. The species showing evidence of infection with more than one serotype were:

In 2 *P. nasuta:* 1 *canicola* group + *medanensis**, 1 *medanensis** + *hyos* + *celledoni*.

In 4 *I. macrourus:* 1 *canicola* group + *hebdomadis* group, 1 *grippotyphosa* + *medanensis**, 1 *pyrogenes* group + *grippotyphosa* + *medanensis**, 1 *grippotyphosa* + *medanensis** + *hyos* + *celledoni*.

In 1 *H. chrysogaster: hyos** + *celledoni*.

In 4 *U. caudimaculatus:* 1 *zanoni** + *pomona*, 1 *canicola* group + *hyos**, 1 *australis** + *pomona*, 1 *zanoni** + *pomona* + *grippotyphosa*.

In 1 *R. s. conatus: pyrogenes* group + *grippotyphosa**.

Isolations indicated by *.

New host records

Isolation and identification of strains extended the proven host range of six serotypes (Table 5). The isolation of *zanoni* from *U. caudimaculatus* and *M. cervinipes* extends the host range of this serotype to all rodents trapped in North Queensland except *H. chrysogaster*.

Maintaining and incidental hosts

The excretion index (Emanuel *et al.* 1964) was calculated for each association between serotype and host where the numbers permitted. The samples were small, but the figures may be considered to give an indication of which animals are the maintaining hosts of the two main serotypes, *grippotyphosa* and *medanensis*, under investigation.

Infections with *grippotyphosa* were recorded from *R. s. conatus* and *I. macrourus*. *R. s. conatus* exhibited an infection rate of 23 %, and an excretion index of 1·0, all animals with evidence of infection being excretors. It could thus be an important maintaining host in the area. On the other hand, *I. macrourus* had an infection rate of 13 % and excretion index of 0·07, so it may well be only an incidental host, as *R. rattus* presumably was in the earlier survey.

Table 5. *New host records of serotypes*

Host species	Serotype
P. nasuta	*medanensis* (2)
I. macrourus	*medanensis* (3)
	grippotyphosa (1)
H. chrysogaster	*hyos* (1)
U. caudimaculatus	*zanoni* (2)
	australis (1)
M. cervinipes	*bindjei* (1)
	zanoni (2)
R. s. conatus	*grippotyphosa* (8)

Figures in parentheses indicate the number of strains isolated.

Infections with *medanensis* were recorded from *P. nasuta* and *I. macrourus*. Their infection rates of 12 and 7 %, respectively, for *hebdomadis* group infections, almost exclusively *medanensis* in this instance, and excretion indices of 1·0 and 0·7, respectively, suggest that both are maintaining hosts. It is noteworthy that here, as in the earlier investigations, no rodents were infected with members of the *hebdomadis* group.

Infection rates for *bindjei* cannot be determined because of serological cross reactions in the *canicola* group. However, *M. lutillus* and *M. cervinipes* inoculated with *bindjei* became urinary carriers (Emanuel *et al.* 1964) and the serotype has been isolated only from these rodents. On these grounds it may be considered that *Melomys* species are the maintaining hosts of the serotype in this area.

DISCUSSION

The ready isolation of *grippotyphosa* and *medanensis* strains from animals in an area where the only human strains were isolated years previously, emphasizes the focal nature of the distribution of some serotypes.

Whilst *R. s. conatus* is an undoubted maintaining host of *australis*, an association responsible for a preponderance of human infections in Division 3 of Mulgrave shire, it appears to play a similar important role in relation to *grippotyphosa* in the

Douglas shire. The bandicoots, *P. nasuta* and *I. macrourus*, here fill the role, as maintaining hosts of *medanensis*, that they play in association with the other *hebdomadis* group serotypes, *kremastos* and *mini*, in more southerly districts of the sugar belt.

There is some serological evidence that both *grippotyphosa* and *medanensis* occur outside the Douglas shire, even in New South Wales. Since 1954, ten patients have submitted multiple specimens of sera which showed titres of 1:300 or more for *grippotyphosa* (unaccompanied by a pattern of cross reaction indicative of infection with other serotypes). Five of these lived on the North Queensland coastal plain, one came from Mackay on the central coastal plain, and four lived and worked in south-eastern Queensland and northern New South Wales. Furthermore, Forbes, Keast, Wannan & Lawrence (1955) reported a series of fifteen bovine sera which contained antibodies to *grippotyphosa*. These sera were collected from cattle in south-eastern New South Wales. Since 1954 also nine patients have had multiple specimens of sera showing titres of 1:300 or more for *medanensis* and lower titres for other serotypes in the *hebdomadis* group. Of these, five lived on the coastal plain north of Innisfail and four lived in south-eastern Queensland. However, no survey of native animals for leptospiral infection has been made by us outside North Queensland.

The isolation of *bindjei* from *M. cervinipes* provides the second recorded rodent host of this serotype in the shire. The animal was trapped in the Vassalini area on Whyanbeel Creek, about 5 miles downstream from the farm on which *bindjei* had been isolated previously from *M. lutillus* and where a human infection had occurred, as recorded in Part I.

Significant infection rates for *pomona* in *I. macrourus* (11 %), *M. cervinipes* (6 %), *U. caudimaculatus* (4 %), and *R. s. conatus* (3 %) were not accompanied by evidence of urinary excretion. The relative frequency of *pomona* infections is lower (22 %) than that (53 %) reported from the Douglas shire by Emanuel *et al.* (1964). *U. caudimaculatus* is a host for a wide range of serotypes in this area, and there is some evidence that it might prove to be a maintaining host for *zanoni*, *hyos* and *australis*.

SUMMARY

Investigations in the Mossman districts of North Queensland showed the maintaining hosts of leptospiral serotype *medanensis* to be *Perameles nasuta* and *Isoodon macrourus* in canefields, secondary woodland and rain forest. *Rattus sordidus conatus* is the maintaining host of *grippotyphosa* in canefields. The host range of six serotypes was extended by the cultural studies.

We would like to express our thanks to Dr G. Alberts, superintendent of Mossman District Hospital, for his co-operation, and also to Miss H. MacDonald and Mr A. Wood for technical assistance.

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J. Hyg., Camb. (1964), **62**, 495
Printed in Great Britain

An improved formate lactose glutamate medium for the detection of *Escherichia coli* and other coliform organisms in water

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(*Received* 2 *June* 1964)

INTRODUCTION

In a previous communication (Gray, 1959) a simple formate lactose glutamate medium was described which, it was suggested, might replace MacConkey broth in the presumptive coliform examination of water. Besides being considerably cheaper, the synthetic medium yielded more isolations of true coliform organisms (and of *Escherichia coli*) with equal speed and with fewer false positive reactions. There was, however, the disadvantage that a proportion of minimally positive tubes could not be recognized until shaking had produced effervescence; and there was the further drawback that the medium was unsuitable for the 44° C. test. Since this medium contained a bare minimum of ingredients it was hoped that suitable small additions might overcome these defects without disturbing the specificity for the coliform group. The present communication describes a modification, devised after a prolonged succession of comparative trials, which surpasses standard MacConkey broth in the presumptive coliform test and is at least its equal in the 44° C. test for *Esch. coli*.

PRELIMINARY COMPARISONS

The formulae for the old and the improved formate lactose glutamate media (single strength) are as follows:

	Formate lactose glutamate (Gray, 1959) (pH 6·7)	Improved formate lactose glutamate (pH 6·7)
Sodium formate	0·25 g.	0·25 g.
Lactose	10 g.	10 g.
L(+)-glutamic acid	5 g.	5 g.
L(+)-arginine mono-hydrochloride	—	0·02 g.
L(−)-aspartic acid	—	0·024 g.
L(−)-cystine	—	0·02 g.
Potassium phosphate (K_2HPO_4)	3 g.	1 g.
Ammonium lactate (50 % w/w solution)	10 ml.	—
Ammonium chloride (NH_4Cl)	—	2·5 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	—	0·2 g.
Calcium chloride ($CaCl_2$)	—	0·2 g.
Ferric citrate scales (M.W. 335)	—	0·1 g.
Thiamin	—	1 mg.
Nicotinic acid	—	1 mg.
Pantothenic acid	—	1 mg.
Brom-cresol purple (1 % alcoholic solution)	1 ml.	1 ml.
De-ionized water	1000 ml.	1000 ml.

The objective in changing from the previous formula was to improve the *quality* of lactose fermentation by the coliform group. In this respect it was important that acid production should become more readily evident, and it would be an additional advantage if the volume of gas evolved could be increased. The preliminary comparisons, therefore, proceeded along two lines: tentative changes in the formula were first tested by minimal inocula of pure cultures of coliform organisms (principally *Esch. coli* itself), and modifications which thus proved successful were then made up in bulk and subjected to a short series of comparisons using unchlorinated water as the inoculum—on the lines of the final tests to be described. The water comparisons had the twofold object of confirming that the modification retained the numerical advantages of the original medium but did not introduce an undue liability to the production of false positive reactions.

Since publication of the previous paper (Gray, 1959) it had become clear that in the preliminary pure-culture experiments the inoculum used (one loopful of an 18 hr. culture) had been much too large. For this reason various projected additions to the original formula of Folpmers (1948) had *appeared* to be unrewarding. Further trials showed that, in order to simulate the conditions prevailing in water analysis, the inoculum should as nearly as possible approach a single viable organism, or for practical purposes three to ten organisms, as established by counts on blood agar done by the method of Miles & Misra (1938). It also became apparent that storage of the organisms in de-ionized water provided an extra stringent test of the synthetic medium and also explained its advantage over MacConkey broth: the presence of bile salt prevented the initiation of growth in a proportion of stored organisms (which grew well enough in the synthetic media) and this proportion increased with increasing time of storage.

It therefore became a regular practice to add 0·02 ml. of an 18 hr. culture to 150 ml. of sterile de-ionized water and to use as the inoculum 0·02 ml. of a suitable dilution. At first the dilution would be of the order of 1 in 100 but lower dilutions were indicated as the organisms died off until the dosage became 0·02 ml. and larger volumes (up to 1 ml.) of the undiluted fluid. To decrease the death rate (which was quite rapid with some of the cultures) sodium thiosulphate was included in the 6 oz. storage bottles used. At first the ordinary water-sampling concentration of approximately 20 p.p.m. was employed, but considerably greater benefit was later obtained from the concentration of 100 p.p.m. recommended by the American Public Health Association (1955) and by Hoather (1957).

The bacterial suspension having been thus prepared and checked, the inoculum was added by dropper to replicate tubes (usually five but sometimes three) of single-strength (*a*) MacConkey broth, (*b*) original formate lactose glutamate medium, and (*c*) one or more of the modifications under test, and also to duplicate plates of blood agar for concurrent viable counts. MacConkey broth was retained throughout as the standard control, but in due course the original formate lactose glutamate medium was replaced in the series by the interim modification so far approved.

By this means it was possible to conduct a very large number of experiments, and to find (*a*) whether any additional substance improved the medium's performance,

and (*b*), if so, the optimal concentration of the substance. In certain instances, as with the more expensive amino acids, we sought not the optimum but the minimum effective concentration.

One of the earliest changes made was to replace ammonium lactate by an equivalent amount of ammonium chloride. The result was a doubling of the gas evolved, suggesting, as had been conjectured, that the lactate by competing with the lactose had interfered with the fermentation. On the suggestion of Burman (1960, pers. comm.) the concentration of potassium phosphate was reduced from 0·3 to 0·1 %. This change did not alter the speed of fermentation but, by removing much of the buffer effect, allowed acid production to become more readily visible. It was found that acid production was further enhanced by the addition of magnesium sulphate. The range of effective concentration was quite wide but after a number of trials it appeared that 0·2 g./l. was optimal. Later on in the trials significant improvements in fermentation were obtained by the addition of calcium and iron. The addition of iron introduced solubility problems. Ferrous sulphate, although beneficial, had to be abandoned since it produced a precipitate either immediately or during incubation. Similar difficulties occurred with ferric chloride. The more soluble ferric citrate provided equal benefit without these difficulties. The addition of manganese did not produce any detectable advantage.

On the question of additional amino acids the observations of previous workers, principally Pinsky & Stokes (1952) and Gest (1954) led us to expect, despite our earlier failure with large inocula, that there should be some advantage obtainable. With the minimal inocula we could detect an immediate benefit with aspartic acid and arginine added singly, but a considerably greater benefit when they were added together. The effect seemed to be greatest when they were added in equimolecular proportions. The original mixture tried was aspartic acid 0·24 g./l. and arginine 0·2 g./l., but for reasons of expense these amounts were successively reduced and an equally good effect was obtainable with one tenth of these quantities. At this stage, a further suggestion from Burman (1960, pers. comm.) was tried, that of doubling the glutamic acid concentration (i.e. to 10 g./l.). In the presence of arginine (0·02 g./l.) and aspartic acid (0·024 g./l.) the extra glutamic acid produced no effect. On the other hand, a halving of the glutamic acid concentration (i.e. to 2·5 g./l.) dramatically impaired the medium; normal function could be restored by greatly increasing the amounts of arginine and aspartic acid, but it was, of course, much more economical to revert to the original concentration of glutamic acid.

Most of the experiments with added minerals were, in fact, conducted with the medium already containing these three amino acids in the amounts listed in the formula. Once the mineral basis had been adjusted, further amino acids were tried. The choice was restricted to the cheaper ones (e.g. cystine, glycine, histidine, leucine, lysine and tyrosine). The only discernible benefit was obtained from cystine alone and it became evident that above a certain concentration (approx. 0·1 g./l.) cystine exerted a strongly inhibitory action. The optimum level to match the other amino acids present appeared to be 0·02 g./l. At this stage the purine bases had also been tested and an almost similar benefit was detected with guanine. Since, how-

ever, the presence of cystine and guanine together conferred no advantage over that of cystine alone, guanine was excluded.

Finally, numerous growth factors were tried including most of the known factors of the vitamin B complex. The effect of thiamin was a noticeable speeding of fermentation; nicotinic acid and pantothenic acid also seemed to confer some benefit. Other factors tested and discarded were nicotinamide, riboflavine, pyridoxine, cyanocobalamin, and para-aminobenzoic acid. The inclusion of nicotinic acid in the formula resulted less from benefits observed at 37° C. than from the observations of Ware (1951) that nicotinamide (or nicotinic acid) was an essential nutrient for some strains of *Esch. coli* to produce fermentation at 44° C.

When the finally developed medium came to be prepared at double strength, problems of insolubility recurred. It was impossible to avoid the formation of a precipitate on heating when the medium contained lactose, cystine, ferric citrate and potassium phosphate in the concentrations listed. The presence of lactose and of phosphate was, of course, obligatory. The precipitate could be avoided by omitting from the formula either cystine or iron, both of which had significantly added to the medium's performance in the single-strength tests. It was eventually found quite practicable to include a precipitation and filtration process during the preparation of the double-strength stock (see Appendix): the optimum performance of the medium was thereby maintained and no further precipitate occurred during sterilization or subsequent incubation.

MATERIALS AND METHODS

These refer to the comparative trials made between standard MacConkey broth and the finally developed medium.

Media used

MacConkey broth (double strength and single strength) was prepared as officially recommended (Ministry of Health, 1956). The peptone (Evans's) was adopted for optimal gas production and the bile salt (Hopkin and Williams's sodium taurocholate) for minimal inhibition. The double-strength medium was distributed in 10 ml. volumes in $6 \times \frac{3}{4}$ in. test tubes and the single-strength medium in 5 ml. volumes in $6 \times \frac{5}{8}$ in. test tubes, all containing Durham tubes.

The improved formate lactose glutamate medium (double and single strength) was prepared as described in the Appendix. Distribution was the same as for MacConkey broth except that for the single-strength medium $6 \times \frac{1}{2}$ in. test tubes were used. Burman & Oliver (1952) recommended this procedure for the original glutamic acid medium but experience at this laboratory suggests that with the new medium the $6 \times \frac{5}{8}$ in. tubes are equally suitable.

Sterilization of media

Both media were autoclaved at 10 lb. for 10 min. Previously, to minimize hydrolysis of the lactose, the synthetic medium had been sterilized by steaming for 45 min. on each of 2 successive days. In the course of this investigation, however,

it was discovered that the lactose in the improved synthetic medium could withstand one autoclaving as well as in MacConkey broth.

Water samples and procedure

(*a*) *Presumptive coliform trial*

All samples of unchlorinated water arriving at the laboratory for routine examination were inoculated into each medium as follows: 10 ml. into each of five tubes of double-strength medium; and 1 ml. into each of five tubes of single-strength medium. The tubes were incubated at 37° C. and inspected after approximately 18, 24 and 48 hr. Those showing production of acid and gas were recorded as presumptive positives.* These were at once further tested by subculture in MacConkey broth for incubation at 44° C. for 24 hr. for gas production, and plating on to MacConkey agar for incubation at 37° C. for 24 hr. in order to provide colonies for any subsequent testing.

Any presumptive positive tube giving a positive fermentation test at 44° C. was regarded as containing *Esch. coli*. When no fermentation occurred at 44° C., several colonies (if there were any) from the MacConkey plate were subcultivated into lactose peptone water. If, after 48 hr. incubation at 37° C., the lactose peptone water showed acid and gas, the presumptive tube was considered to contain true coliform organisms other than *Esch. coli*. If there were no colonies on the MacConkey plate or if, after 48 hr. incubation, the lactose peptone water failed to show acid and gas, the presumptive positive reaction was classified as a false positive reaction, i.e. a presumptive positive tube from which no lactose-fermenting coliform organisms could be isolated.

(*b*) *Comparative trial of* 44° C. *test for* Esch. coli

Since the presumptive coliform trial showed the synthetic medium to produce at all periods of incubation a greater yield both of coliform organisms and of *Esch. coli*, it was decided, in this second comparison, to perform the preliminary presumptive test only in the synthetic medium. During this trial all samples of unchlorinated water arriving at the laboratory for routine examination were inoculated into the synthetic medium in the ordinary way (50 ml. and 5 × 10 ml. volumes into double-strength and 5 × 1 ml. volumes into single-strength medium). The bottles and tubes were incubated at 37° C. and inspected after approximately 18, 24 and 42 hr. The routine tests were usually started at or about 4 p.m.; the 18 hr. and 42 hr. periods occurred therefore at or about 10 a.m. Tubes showing production of acid and gas were recorded as presumptive positives and those occurring at 18 and 42 hr. were subcultured for the 44° C. tests at 10 a.m. Those showing acid and gas first at 24 hr. were subcultured at 4 p.m. and then the primary tubes were placed in the refrigerator until the following morning. From these a second sub-

* In accordance with standard practice the minimum criterion for a 'positive' MacConkey tube was the presence of acid, together with enough gas to fill the concavity of the Durham tube. With the synthetic medium, however, a reliable minimal criterion was clearly detectable acid together with any gas (produced, if necessary, as effervescence after shaking).

culturing was then made at 10 a.m. in order to provide for a 6 hr. reading during working hours. Preliminary experiments suggested that this period of refrigeration did not affect the subsequent 6 hr. result.

From each presumptive positive tube a loopful of medium was transferred into single-strength (*a*) MacConkey broth and (*b*) improved formate lactose glutamate medium. The duplicate tubes were then placed in the 44° C. water bath and inspected (for gas production) (1) after 6 hr. and (2) after 24 hr.

(*c*) *General P.H.L.S. trial*

It was considered advisable to check the Newport results, and 27 other P.H.L.S. laboratories undertook to participate in a comparative trial between their regular standard water medium (MacConkey broth or Teepol broth) and the synthetic medium (which was prepared in Newport and distributed to the other laboratories). Each participating laboratory agreed to examine approximately 30 unchlorinated water samples as follows: 5×10 ml. volumes and 5×1 ml. volumes were inoculated into appropriate tubes of both media which were incubated at 37° C. and inspected at 18 and/or 24 hr. and 48 hr. Each presumptive positive tube (without necessarily testing for false positive results) was then subcultured into fresh tubes of both media which were held at 44° C. for 24 hr. and then inspected for gas production.

RESULTS

Presumptive coliform trial at Newport

Over a period of 4 months 100 routine samples of unchlorinated water were examined in the manner described. The cumulative results are shown in Table 1, where it can be seen that, as regards both true coliform organisms and those subsequently identified as *Esch. coli*, the numbers of isolations appearing by 18, 24 and 48 hr. were all noticeably greater in the synthetic medium than in MacConkey broth. In comparing these figures it must be borne in mind: (*a*) that in order to minimize the suppressive effect of bile salt on the coliform group (see P.H.L.S. Water Sub-committee, 1958; Gray, 1959) a relatively non-inhibitory brand of sodium taurocholate was specially chosen; and (*b*) the higher figures obtained in the synthetic medium were virtually unassociated with false positive results.

Table 1. *Reactions produced by* 100 *samples of unchlorinated water examined at Newport, Monmouthshire*

Medium	No. of false positive reactions appearing by			No. of true coliform reactions appearing by			No. of *Esch. coli* producing a presumptive positive reaction by		
	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
MacConkey broth	0	1	18	128	151	517	115	123	127
Improved formate lactose glutamate medium	0	0	1	140	189	651	136	178	199

Newport comparative trial of 44° C. *test for* Esch. coli

Over a period of 7 months 1099 presumptive positive tubes arising in the synthetic medium from 156 samples of unchlorinated water were tested in duplicate as already described. The results are shown in Table 2, where it can be seen that in the 44° C. test both media behaved almost identically. It is noteworthy that from the three different types of presumptive positive tubes (18, 24 and 48 hr.) the 6 hr. 44° C.-positive tubes are as numerous in the synthetic as in the standard medium.

Table 2. 44° C. *test for* Escherichia coli

(Comparison between MacConkey broth and improved F.L.G. medium—results arising from 156 samples of unchlorinated water examined at Newport, Mon.)

Presumptive positive reactions detected in improved F.L.G. medium		No. producing fermentation at 44° C. in			
		MacConkey broth by		Improved F.L.G. medium by	
Time of appearance (hr.)	No.	6 hr.	24 hr.	6 hr.	24 hr.
18	536	532	535	533	536
24	121	70	79	70	82
42	442	17	35	18	42
Totals	1099	619	649	621	660

General P.H.L.S. trial

The results of this trial are shown in Table 3. Although there are undoubted fluctuations from laboratory to laboratory, the aggregated totals confirm the trend shown in Newport.

First, with regard only to the presumptive positive results, the totals by 18 and 24 hr. were slightly greater and by 48 hr. considerably greater in the synthetic than in the standard medium. The final increase (3054 − 2830 = 224) represents an 8 % improvement upon the performance of the standard medium. Since false positive results are much less common in the synthetic than in the standard media it is to be expected that full elimination of these would have shown an even greater improvement.

Secondly, with regard to the presumptive positive results which were shown by the 44° C. test to contain *Esch. coli*, the positive 37° C. tubes in each medium were tested in both media at 44° C.; there were therefore 4 ways in which positive 44° C. tests could be obtained:

(1) presumptive positives in standard water medium producing positive 44° C. results in standard water medium 'S.W.M./S.W.M.';

(2) presumptive positives in standard water medium producing positive 44° C. results in improved F.L.G. medium 'S.W.M./I.F.L.G.';

(3) presumptive positives in improved F.L.G. medium producing positive 44° C. results in standard water medium 'I.F.L.G./S.W.M.';

Table 3. *Reactions produced by 785 samples of unchlorinated water examined in 27 P.H.L.S. laboratories*

Laboratory	No. of samples examined in duplicate	No. of samples giving positive results in at least one medium	No. of presumptive coliform reactions appearing in: Standard water medium by 18 hr.	24 hr.	48 hr.	Improved F.L.G. medium by 18 hr.	24 hr.	48 hr.	No. of *Esch. coli* producing a presumptive positive reaction in: S.W.M./S.W.M. by 18 hr.	24 hr.	48 hr.	S.W.M./I.F.L.G. by 18 hr.	24 hr.	48 hr.	I.F.L.G./S.W.M. by 18 hr.	24 hr.	48 hr.	I.F.L.G./I.F.L.G. by 18 hr.	24 hr.	48 hr.
Bath	30	18	28	28	64	27	27	65	27	27	28	27	27	29	27	27	30	27	27	29
Birmingham	38	21	—	66	74	—	92	109	—	66	68	—	66	68	—	91	96	—	92	97
Bradford	32	22	—	94	112	—	101	116	—	92	98	—	92	98	—	97	104	—	97	104
Brighton (T)	30	25	—	110	137	—	120	144	—	89	89	—	88	88	—	96	97	—	95	96
Bristol	30	24	56	60	138	51	57	127	49	52	58	49	52	58	45	49	60	44	48	59
Cambridge (T)	26	10	10	16	48	7	15	46	9	11	11	9	11	11	5	7	7	5	7	7
Cardiff	23	22	—	104	138	—	109	144	—	102	123	—	97	106	—	98	103	—	92	98
Carlisle	30	19	—	67	87	—	77	105	—	67	68	—	67	68	—	76	76	—	76	76
Carmarthen	21	17	—	62	101	—	58	135	—	31	33	—	28	30	—	30	33	—	28	31
Conway	34	29	85	85	198	81	81	204	66	66	75	65	65	72	73	73	93	73	73	91
Dorchester	29	20	—	98	132	—	100	129	—	60	85	—	60	85	—	64	82	—	61	79
Hull	14	14	—	79	119	—	43	116	—	75	88	—	76	90	—	42	94	—	42	96
Leicester	23	19	—	76	136	—	71	134	—	66	68	—	66	68	—	62	69	—	62	69
*Manchester (T)	35	29	134	155	164	126	152	191	130	136	136	130	136	136	125	145	146	125	145	146
*Northallerton	29	23	40	66	101	65	83	122	25	31	34	33	42	45	39	45	47	40	46	48
Norwich (T)	30	19	30	51	122	29	67	135	23	25	28	23	25	30	23	31	33	20	29	33
Nottingham	59	9	10	22	29	10	28	44	10	21	21	10	21	21	10	26	27	10	26	27
Plymouth	30	25	—	88	138	—	80	149	—	86	98	—	86	98	—	77	101	—	77	101
Portsmouth (T)	13	13	35	36	52	35	35	47	32	32	32	32	32	32	31	31	32	32	32	34
Preston	28	18	32	32	57	57	57	84	27	27	36	30	30	39	48	48	55	52	52	61
Salisbury	27	9	3	5	67	1	7	81	—	—	39	—	—	45	—	—	42	—	—	50
Shrewsbury (T)	30	19	—	35	108	—	29	95	—	24	26	—	24	26	—	19	26	—	19	26
Southend	30	27	53	91	130	45	73	151	38	42	48	38	42	48	40	42	52	39	41	51
Stafford	30	17	21	34	84	10	17	64	9	9	9	9	9	9	9	9	9	9	9	9
Taunton	30	19	—	8	88	—	11	99	—	—	17	—	—	32	—	—	20	—	—	30
*Wakefield	28	20	52	72	99	51	64	114	42	55	57	42	55	57	41	51	51	41	51	51
Worcester	26	17	—	47	107	—	47	104	—	37	38	—	37	38	—	41	42	—	41	42
Totals	785	524	589	1687	2830	595	1701	3054	487	1329	1511	497	1334	1527	516	1377	1627	517	1368	1641

* False positive reactions checked and excluded.
Most laboratories used MacConkey broth as their standard water medium; those using Teepol broth are marked (T).

(4) presumptive positives in improved F.L.G. medium producing positive 44° C. results in improved F.L.G. medium 'I.F.L.G./I.F.L.G.'.

If the aggregated totals are compared, it will be seen that method (1) (S.W.M./S.W.M.) yields the lowest totals of *Esch. coli* at all three periods; that method (2) (S.W.M./I.F.L.G.) shows a slight increase, method (3) (I.F.L.G./S.W.M.) a still greater increase and method (4) (I.F.L.G./I.F.L.G.) the highest figures of all at 18 and 48 hr. As was to be expected from the greater presumptive yield in the synthetic medium, the most noticeable increases in the *Esch. coli* totals occur where the synthetic medium was used for the presumptive test: 1627 and 1641 as against 1511 and 1527.

DISCUSSION

The results in Newport (Tables 1 and 2) and in the other 27 areas, considered as one whole (Table 3, column totals), suggest that from non-chlorinated water (1) improved formate lactose glutamate (I.F.L.G.) produces more isolations both of presumptive coliform organisms and of *Esch. coli* than does standard MacConkey broth (or its derivative Teepol broth) and (2) the increased isolations are obtained without loss of speed: only slight increases are visible by 18 and 24 hr. but noticeable increases are obtained by 42–48 hr.

False positive reactions

The work at Newport additionally suggests that, so far as non-chlorinated water is concerned, the synthetic medium produces so small a proportion of false positive reactions that presumptive positive reactions, even at 48 hr., can be reliably considered to contain true coliform organisms. It is, perhaps, unfortunate that in the wider trial only three of the twenty-seven laboratories tested for false positive reactions; but they were not asked to do so, as this is not customarily done in the routine examination of non-chlorinated water, and the object of the trial was to simulate standard routine as closely as possible. The three laboratories which included the extra tests produced the following totals of false positives: (*a*) from MacConkey (or Teepol) broth 5, and (*b*) from the synthetic medium 7 (all obtained at Manchester). As compared with their total isolations of true coliform organisms, (*a*) 364 and (*b*) 427, the figures for false positives are too small to invalidate the usual practice of concluding that, with non-chlorinated water, presumptive coliform reactions can be taken to represent the presence of coliform organisms.

The problem of false positive reactions, however, has always been principally associated with chlorinated waters, and the chief reason for this is the survival (after chlorination) of *Cl. welchii* and similar sporing anaerobes which readily ferment lactose in MacConkey broth (Taylor, 1949; P.H.L.S. Water Sub-committee, 1953) and in other conventional water media incorporating peptone (Teepol broth, American lactose broth, and lauryl tryptose broth—see Jebb, 1959). The original formate lactose glutamate medium (Gray, 1959), like its predecessor lactose glutamic acid (Burman & Oliver, 1952; P.H.L.S. Water Sub-committee, 1958), produced an almost negligible proportion of false positive reactions even from chlorinated waters. It had been found in Newport that neither of these media

would support the growth of *Cl. welchii* even after heavy inocula. In the course of modifying the 1959 medium we repeatedly checked that the extra nutrients added did not destroy this advantage; no strain of *Cl. welchii* has been found to ferment or even grow in the final medium. It was, therefore, hoped that with this medium the problem of false positives would become virtually eliminated, and this hope is encouraged by recent experience at Newport. Here, since 1 May 1963, improved formate lactose glutamate has been exclusively used for routine water analysis and every one of the few presumptive positive reactions arising from chlorinated water has been fully tested. So far, after 12 months, 1224 chlorinated samples have been examined of which 1111 were completely negative. From the remaining 113 samples, 396 presumptive positive tubes were obtained. Each of these proved to contain true coliform organisms (and 170 *Esch. coli* itself). In other words, not one false positive reaction has yet been discovered from chlorinated water samples examined in I.F.L.G. at Newport.

In the Metropolitan Water Board Laboratories, however, where a different modification of formate lactose glutamate has been in routine use since January 1962 (Taylor, 1964), certain chlorinated waters—principally river-derived supplies—have been found to yield, particularly in the winter months, a substantial proportion of false positive reactions after 48 hr. incubation. With experience these became readily recognizable from true reactions by the poor degree of acid and the small quantity of gas evolved, and are now being discarded on sight. The causative organisms are *aerobic* spore-bearers, and five representative cultures were recently sent to Newport (Burman, 1964, pers. comm.). Heavy inoculation of each culture into I.F.L.G. produced by 48 hr. (and sometimes sooner) indisputable fermentation, i.e. detectable acid associated with adequate gas. This is not to say that similar appearances would have been produced in the I.F.L.G. medium by the smaller numbers to be expected in the natural conditions of water examination; the Newport medium is now being tested by the M.W.B. laboratories. Only further and much more general experience will determine whether this is a problem peculiar to London (or the M.W.B. version of F.L.G.) or whether it may be met in other parts of the country such as, perhaps, Manchester. It is certainly no problem at present in Monmouthshire, where most of the chlorinated supplies come from surface waters.

Interpretation of routine water analysis

From the work at Newport and elsewhere it would seem reasonable to suggest that presumptive positive reactions from non-chlorinated water appearing in I.F.L.G. by 18 hr. might be deemed without further testing to contain *Esch. coli*. In this way, without serious error, the 44° C. test could be reserved for the many fewer presumptive positive reactions arising at 24 and 48 hr. The saving in labour would be worth considering.

In the case of chlorinated waters, which should, of course, always ideally be totally negative, any presumptive positive reaction would continue to require (1) checking to eliminate false positives and (2) differentiation into *Esch. coli* or other true coliform organisms.

Observations regarding the new medium

It has been the intention in Newport to improve the original F.L.G. medium so as to provide the coliform group, and *Esch. coli* particularly, with the optimum ingredients within the limits of expense and practicability for maximum growth and lactose-fermentation, while as far as possible limiting the available carbon and energy source to lactose. Having been thus 'tailor-made' for the coliform group, the medium requires no inhibitory substance. Non-lactose-fermenters are substantially inhibited by the lack of a suitable energy source: herein lies the advantage over glucose glutamic acid. On plates of solid I.F.L.G., containing ten times the quoted concentration of brom-cresol purple indicator, this effect can be seen. Yellow coliform colonies attain after overnight incubation something approaching the normal size of 2 mm. diam., usually with a surrounding yellow zone of diffused acid; non-lactose-fermenters (e.g. salmonellae) under the same conditions appear as purple pin-point colonies clearly distinguishable by colour and size, so long as colonies of both types are reasonably discrete. Proteus can produce purple pin-point colonies but is unable to swarm. Enterococcoci are unable to grow at all.

Whilst it is not suggested that I.F.L.G. has any claim to replace MacConkey agar or similar media in general bacteriology, as for example routine examination for faecal pathogens, it is hoped that I.F.L.G. agar may serve as a basis for the coming method of routine water examination—membrane filtration. So far, only a few pure-culture experiments have been made but these suggest that the membrane should be incubated over a plate of solid medium rather than over the usual pad impregnated with liquid medium. By the former method *Esch. coli* colonies, after overnight incubation at 37° C., reach full normal size (2–3 mm. diam.) and present a bright yellow colour, whereas non-lactose-fermenters such as Proteus produce only pin-point colonies, either colourless or pale violet, which are almost invisible to the naked eye. There is, therefore, reason to hope that the coliform count would become much easier to read than with MacConkey agar and neutral red (or phenol red), where pink colonies have to be distinguished from yellow.

Finally, as regards expense, the original F.L.G. medium was found in 1959 to be 2·5 times cheaper than MacConkey broth. The I.F.L.G. medium has small amounts of extra ingredients but is not significantly more expensive than F.L.G. Meanwhile, however, the prices of the biological ingredients have so increased that the cost of the cheapest MacConkey broth is now more than four times, and the cost of Teepol broth made with a good peptone is more than three times, that of I.F.L.G. The increasing use made of bile salt for pharmaceutical purposes will almost certainly lead to further scarcity and higher prices, so that the saving offered by the synthetic medium should increase still further. If, in addition, the use of membrane filtration permits 100 ml. of water to be examined by 10 ml. of single-strength agar medium instead of 100 ml. of double-strength fluid medium, the economies would be very considerable.

SUMMARY

A chemically defined fluid medium—improved formate lactose glutamate—is described of which the cost is less than a quarter that of standard MacConkey broth and which is at least equally suitable for routine bacteriological examination of water. It has actual advantages in that the medium after fermentation is clearer and that, therefore, acid and gas production is more easily seen; moreover more isolations of both coliform bacteria and of *Esch. coli* are obtained with fewer false positive reactions. Trials with a solid version of the medium suggest that the relative freedom from false positive reactions arises from the failure of the medium to grow *Cl. welchii*—one of the commonest causes—and enterococci, another suspected participant. In fact, without any inhibitory agent the medium appears to be virtually specific for the coliform group and particularly favours *Esch. coli*. It remains to be seen whether certain aerobic spore-bearers found from time to time in surface waters may, to some slight extent, interfere with the almost complete specificity so far discovered.

The medium is recommended in place of MacConkey broth and other peptone-containing media, not only for the prospect of better results, in that suppression of the coliform group does not occur, but also for cheapness and for freedom from the inevitable variability associated with media incorporating peptone and, particularly, bile salt. It is hoped that this medium will shortly become available commercially in dehydrated form at a price still considerably less than that required to make up MacConkey broth from its constituents.

Some labour-saving curtailments in the routine of water examination are also suggested.

It is hoped not only that the fluid medium will supplant MacConkey broth for the dilution method of water analysis but that the solid medium, without material alteration, will also prove suitable for water examination by membrane filtration.

I am deeply grateful to my laboratory staff, particularly Mr J. H. Evans, F.I.M.L.T., and Mr G. H. Lowe, F.I.M.L.T., for undertaking so much of the work when routine work was particularly heavy, and for many valuable suggestions. My thanks in addition are due to Mrs Peggy Johnson and Mrs Marilyn Childs, who made up the medium, and also to the directors of the participating P.H.L.S. laboratories not only for having the work carried out but for helpful comments.

APPENDIX

Preparation of improved formate lactose glutamate medium

The formula for the single-strength medium has already been given. In practice it is convenient to make 10 l. of double-strength stock solution containing all the ingredients except the lactose and thiamin (to minimize loss by heat) and indicator. It is advisable first of all, however, to make the following stock solutions:

(*a*) Calcium chloride ($CaCl_2$), 25 g.; de-ionized water, 500 ml. Clarify by adding 0·4 ml. of concentrated hydrochloric acid and sterilize by autoclaving at 10 lb. for 10 min.

(*b*) Thiamin, 0·1 g.; de-ionized water, 100 ml. The contents of 1 ampoule (100 mg.) are added aseptically to 99 ml. of *sterile* de-ionized water. (Do not heat.) This solution should be kept in the refrigerator and should be discarded after 6 weeks.

(*c*) Brom-cresol purple, 1 g.; absolute alcohol, 100 ml.

Stock double-strength solution

Place 9 l. of de-ionized water in a stainless-steel bucket and heat. Whilst this is heating, weigh out into 4 separate flasks:

(1) L(+)-arginine mono-hydrochloride	0·4 g.
L(−)-aspartic acid	0·48 g.
(2) L(−)-cystine	0·4 g.
(3) Ferric citrate scales (M.W. 335)	2 g.
(4) Nicotinic acid	0·02 g.
Pantothenic acid	0·02 g.

From a separate litre of de-ionized water are removed measured volumes for dissolving the contents of each flask. In the case of flask (4) the contents are readily dissolved by 5 ml. of cold water. For flasks (1) and (3) 50 and 100 ml. volumes, respectively, together with heating are required. In order to dissolve the cystine in flask (2) it is necessary to use 100 ml. of water, together with heat and the addition of 10 ml. of 5N-sodium hydroxide.

To the 9 l. of water (kept hot in the steamer) add the L(+)-glutamic acid (100 g.) and approximately 140 ml. of 5N-sodium hydroxide. While the bulk is cooling add:

Potassium phosphate (K_2HPO_4)	20 g.
Ammonium chloride (NH_4Cl)	50 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	4 g.
Sodium formate	5 g.

Now add the contents of the four flasks and rinse each flask (with water from the spare litre) into the bucket. Add 80 ml. of the 5% solution of calcium chloride. Finally, discard from the spare litre flask 230 ml. (to compensate for the 80 ml. of calcium chloride solution and the 150 ml. of sodium hydroxide added) and add the remaining water to the bulk.

Allow the bulk to cool and bring the pH to 7·5. Place the bucket on a gas ring and boil for at least 3 min. Filter while hot through Green's 904½ filter-paper. Adjust the pH to 6·8, bottle in 500 ml. amounts and autoclave at 10 lb. for 10 min.

For use

To each 500 ml. as required add lactose (10 g.) and (with aseptic precautions) 1 ml. of the 0·1% solution of thiamin. (For single-strength medium now dilute with an equal volume of de-ionized water.) Check that the pH is 6·8, add the brom-cresol purple solution (1 ml. for each 500 ml. of double-strength or 1000 ml. of single-strength medium), distribute into bottles and tubes, and sterilize by autoclaving at 10 lb. for 10 min. only. The final pH after autoclaving is 6·7.

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Printed in Great Britain

Polyvinyl-pyrrolidone-iodine: an assessment of antibacterial activity

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(*Received* 10 *June* 1964)

As a general disinfectant, iodine is one of the most powerful substances known. It has, however, well-known disadvantages such as irritation of the skin and mucous membranes, staining of the skin and fabrics, and a liability to cause iodism on continued use. In an attempt to overcome these disadvantages, Shelanski & Shelanski (1956) used a combination of iodine with polyvinyl pyrrolidone (PVP-I). This complex is known to possess considerable power of disinfection and shows promise as a skin disinfectant.

Disinfection of the skin as a preparatory measure in surgical procedures still presents many problems, especially in babies. This is particularly the case when surgery has to be practised in an area where even commensal organisms such as *Staphylococcus albus* can cause infection, e.g. in the repair of congenital deformities. The present investigation was designed to explore the possibility of using iodine in the form of the complex with polyvinyl pyrrolidone for this purpose.

METHODS

In these studies the following two PVP-I preparations were investigated for their activity against micro-organisms.

PVP-I aqueous solution (PVP-I sol.)	
PVP-I	1·0 % available iodine
Glycerol	1·0 % (v/v)
Nonyl phenoxy polyoxyethylene ethanol*	0·25 % (v/v)
Buffer, disodium phosphate/citric acid	
PVP-I surgical scrub (PVP-I scrub)	
PVP-I	0·75 % available iodine
Lauric acid diethanolamine condensate*	4·0 % (w/v)
Ammonium alkyl phenoxy polyoxyethylene glycol sulphonate*	25·0 % (v/v)
Sodium hydroxide and hydrochloric acid to adjust pH to 4·6	

Action of PVP-I on vegetative micro-organisms

Non-spore-forming bacteria

An inoculum of 0·3 ml. of a 24 hr. nutrient broth culture of each of the test organisms was added to 3 ml. of PVP-I sol. at 20° C., giving a final bacterial concentration of approximately 1×10^8 organisms/ml. Standard 4 mm. loop samples were taken at 15, 30 and 45 sec. and at 1, 2, 3, 4, 5 and 10 min. into 3 ml. of

* Surface active agents.

nutrient broth containing sodium thiosulphate (1 %) to neutralize any free iodine present. At this concentration sodium thiosulphate did not affect the growth of the test organisms in broth controls. All transplants were incubated for 3 days at 37°, then subcultured on to nutrient agar and read after overnight incubation. Modifications of this technique were carried out to investigate the effect of the presence of biological fluids and other factors described in the text.

Spore-forming bacteria

Vegetative cells of spore-forming bacteria were obtained by centrifuging 72 hr. broth cultures of the organisms, removing the supernatant medium, then adding the same volume of fresh medium and incubating for 4 hr. at 37° C. All clostridia were grown in Robertson's cooked meat medium and aerobic bacilli in nutrient broth containing soil extract (1 %). The organisms were then tested against PVP-I sol. as described for non-spore-forming bacteria above. All transplants of clostridia were made into Robertson's cooked meat medium containing 1 % sodium thiosulphate and of bacilli into nutrient broth containing 1 % sodium thiosulphate. These were incubated for 3 days at 37° C., subcultured on blood agar anaerobically for clostridia and nutrient agar aerobically for bacilli, then read after overnight incubation.

Fungal hyphae

The hyphae from centrifuged 48 hr. Czapex Dox broth cultures (3 ml.) were used as inocula. To the hyphae after the removal of the supernatant medium 3 ml. of PVP-I sol. was added. Standard loop samples were taken as above on to Czapex Dox agar containing 1 % sodium thiosulphate; all transplants were incubated at 25° C. for 7 days.

Action of PVP-I on spores

Bacterial spores

Seventy-two hour broth cultures of the test-sporing bacteria were used after microscopic examination to ensure that spores were present. Inocula (0·3 ml.) of these cultures were added to 3 ml. PVP-I sol. at 20° C.; standard loop samples were taken at 5, 10, 20, 30, 60, 120, 150 and 180 min. and then at hourly intervals to 5 hr. with a final transplant after 17 hr. All the test bacteria were grown in media as for vegetative cells of spore formers and incubated as in this method. This test was repeated for comparison with the following solutions of standard antiseptics at dilutions of 1/1, 1/10, 1/100 and 1/1000: chlorhexidine, 0·5 % (w/v) aqueous; Lugol's iodine; lysol, 1/20 aqueous; potassium permanganate, 10 % (w/v).

Fungal spores

Spores from 7-day plates of the test fungi were suspended in 3 ml. of sterile water. The spore suspension (0·3 ml.) was added to 3 ml. PVP-I sol.; sampling, incubation and blanks were as for fungal hyphae.

Action of PVP-I on bacterial suspensions in fats and oils

The following lipids were used: glycerol triacetate, glycerol tributyrate, glycerol trilaurate, glycerol trioleate, glycerol tripalmitate, glycerol tristearate, glycerol monoricinoleate, arachis oil, coconut oil, olive oil. To 3 ml. of the sterile fat or oil was added 0·3 ml. of a 24 hr. broth culture of the test organism. The resulting mixture was well shaken for 5 min. and 0·3 ml. of the emulsion was added to 3 ml. of PVP-I sol.; sampling and incubation were as for non-sporing bacteria. As some emulsions tended to separate on addition to PVP-I sol., sampling was done carefully to ensure the transference of a fat droplet each time. The individual fats and oils were also allowed to remain in contact with each test organism without PVP-I sol. for 1 hr., and then subcultured into nutrient thiosulphate broth to exclude inhibition by the lipid. PVP-I sol. without the test fats or oils was also tested under similar conditions. This was repeated using PVP-I scrub and aqueous 0·5 % chlorhexidine (w/v).

Sterilization of glass, plastic and metal surfaces

PVP-I was compared with the following standard disinfectants: acetic acid, 5 % (v/v) aqueous; chlorhexidine, 0·5 % (w/v) aqueous; chlorhexidine, 0·5 % (w/v) alcoholic; dettol, 1/20 aqueous; Lugol's iodine; lysol, 1/20 aqueous; sterile distilled water.

Sterile glass and plastic Petri dishes were infected with 0·2 ml. of an overnight culture of the test organism and allowed to dry for 3 hr. The surfaces were then flooded with the test solutions; after 10 min. the solutions were tipped off and the surfaces rinsed gently in distilled water. The plates were then flooded with 6 ml. of nutrient broth and incubated for 24 hr. at 37° C. in a desiccator with a beaker of water to prevent drying. After incubation, the broths were subcultured on nutrient agar plates and incubated overnight, any growth being noted. This was repeated but the surfaces were covered with a thin layer of agar before contamination. Sterile aluminium foil (2·5 × 1·5 mm.), infected by dipping in a 24 hr. culture of the test organism, was then tested similarly. The foil was placed in 10 ml. nutrient broth and incubated. To determine the speed of action of PVP-I sol., infected foil was placed in the PVP-I (1/1 and 1/10) and foil samples withdrawn at 15, 30 and 45 sec. and 1, 2, 3, 4, 5 and 10 min.

Action of PVP-I on Trichomonas

Trichomonas vaginalis was washed from a swab into 5 ml. of sterile saline at 37° C. The resulting suspension was centrifuged; the sediment containing living *Trichomonas* was treated as follows:

(1) A slide preparation in saline was kept as control.

(2) A slide preparation in 1/20 PVP-I made up as a 'Douche' containing equal volumes of both preparations was examined microscopically, death being taken as the point when all movement of the organisms ceased.

Residual bactericidal activity of PVP-I after neutralization with sodium thiosulphate

The iodine in a 10 ml. sample of PVP-I sol. was neutralized by titration with sterile 1 % sodium thiosulphate, the volume used was noted, and this volume of sterile distilled water was added to a further 10 ml. of PVP-I sol. A 3 ml. sample of each solution was taken and the remainder diluted 1/10 to 1/100,000 also in 3 ml. final volumes. To each solution and its dilutions was added 0·3 ml. of a 24 hr. broth culture of *Staphylococcus aureus*. Standard loop samples were taken at 3, 4, 5, 7, 9 and 10 min., transferred to 3 ml. nutrient thiosulphate broth, incubated at 37° for 24 hr., plated on to nutrient agar and re-incubated. A broth blank was set up as before and also one for the neutralized PVP-I sol. containing the same concentration of sodium thiosulphate.

Action of PVP-I on the skin flora

Unless stated otherwise, all samples of the skin flora in these studies were taken by a modification of the velvet pad replica-plate technique (Lederberg & Lederberg, 1952). The velvet pad was stuck on to aluminium foil to allow it to remain in apposition to the area being swabbed. Before use, the sterile pad was moistened in nutrient broth, then pressed over the area to be sampled; it was then pressed on to a horse blood agar which was incubated at 37° C. overnight; any colonies formed were counted, mapped and identified.

RESULTS

Action of PVP-I on vegetative micro-organisms

PVP-I sol. (1/1 and 1/10) killed the majority of vegetative test organisms within 30 sec. (Table 1), though some strains of *Staph. aureus* required 3 min. to be killed. The presence of 5 % of serum or 10 % of citrated whole blood in the antiseptic delayed the lethal action of PVP-I sol. on one strain of *Staph. aureus* by 15 sec. PVP-I sol. was tested at a dilution of 1/10 against 160 strains of vegetative bacteria, all isolated from clinical sources, using 0·3 ml. inocula containing approximately 10^{12} organisms/ml. All the test organisms were killed within 10 min., but at 5 min. 7 organisms (5 strains of *Staph. aureus*, 1 coliform and 1 *Pseudomonas pyocyanea*) remained alive. PVP-I sol. was however uniformly bactericidal at 1/100 dilution in 1 min. to lighter suspensions (0·3 ml.) of 10^9 organisms/ml. washed 24 hr. cultures (Table 1).

Action of PVP-I on spores

PVP-I sol. did not kill any of the bacterial spores tested within 17 hr. Lugol's iodine was the only test solution to show sporicidal activity, killing at all dilutions. Fungal spores were all killed within 90 min. by PVP-I sol. (Table 2).

Action of PVP-I on bacterial suspensions in fats and oils

The addition of tripalmitin, tristearin and, to a less extent, various other fats and oils caused a 3- to 20-fold delay in the bactericidal action of PVP-I, whether in aqueous solution or formulated as a 'scrub'.

The presence of the fats did not affect the growth of the test organisms after 1 hr. incubation. The interfering effect of these fats was stronger against PVP-I than against chlorhexidine (Table 3).

Table 1. *Activity of PVP-I against vegetative micro-organisms*

Test organisms (vegetative)	No. of strains tested: At all dilutions	No. of strains tested: At 1/10 only	Control	PVP-I sol. 1/1	PVP-I sol. 1/10	PVP-I sol. 1/100*	PVP-I sol. 1/1000*
Esch. coli	2	9	G	K	K	K	G
Proteus sp.	2	10	G	K	K	K	G
Ps. pyocyanea	2	5	G	K	K	K	G
Salm. typhi-murium	1	2	G	K	K	K	G
Shigella sonnei	1	2	G	K	K	K	G
Staph. albus	2	27	G	K	K	K	G
Staph. aureus	4	85	G	K	K	K	G
Staph. aureus	1	6	G	K3	K3	K3	G
Haem. Streptococcus							
Group A	1	6	G	K	K	K	G
Group B	1	1	G	K	K	K	G
Group C	1	1	G	K	K	K	G
Group D	1	1	G	K	K	K	G
B. cereus	1	—	G	K	K	—	—
B. subtilis	1	—	G	K	K	—	—
Cl. septicum	1	—	G	K	K	—	—
Cl. sporogenes	1	—	G	K	K	—	—
Cl. tetani	1	—	G	K	K	—	—
Cl. welchii	1	—	G	K	K	—	—
Aspergillus flavus	1	—	G	K	K	—	—
A. niger	1	—	G	K	K	—	—
Candida albicans	2	—	G	K	K	K	G
Penicillium	1	—	G	K	K	*	*

* Washed culture used as inoculum.

G, Normal growth as in controls; K, no growth after 30 sec.; K3, no growth after 3 min.

Table 2. *A comparison of antibacterial agents on bacterial and fungal spores*

Test sporing organism	Chlorhexidine 0·5% aqueous	Lugol's iodine	Lysol 1/20 aqueous	Potassium permanganate 10%	PVP-I sol.	Control
			Bacterial spores			
Bacillus cereus	G17	K180	G17	G17	G17	G
B. subtilis	G17	K180	G17	G17	G17	G
Clostridium septicum	G17	K180	G17	G17	G17	G
Cl. sporogenes	G17	K180	G17	G17	G17	G
Cl. tetani	G17	K180	G17	G17	G17	G
Cl. welchii	G17	K180	G17	G17	G17	G
			Fungal spores			
Aspergillus flavus	—	—	—	—	K15	G
A. niger	—	—	—	—	K90	G
Penicillium	—	—	—	—	K90	G

G, Normal growth as in controls; G17, growth after 17 hr.; K15, no growth after 15 min.; K90, no growth after 90 min.; K180, no growth after 180 min.

Sterilization of glass, plastic and metal surfaces

Of the 7 agents tested, all killed the test organism *Ps. pyocyanea* within 10 min. on clean surfaces. In the presence of agar, 1/20 Dettol was the only agent which failed to kill the test organism within 10 min. PVP-I sol. (1/1 and 1/10) killed the test organism on metal surfaces in 5 min.

Table 3. *Action of PVP-I on bacteria suspended in fats and oils*

	Mean time (min.) for 100 % bactericidal effect					
	Aqueous PVP-I		PVP-I scrub		Chlorhexidine aqueous 0·5 %	
Lipid vehicle for test organism	*Staph. aureus*	*Ps. pyocyanea*	*Staph. aureus*	*Ps. pyocyanea*	*Staph. aureus*	*Ps. pyocyanea*
Blank	3	< 0·5	3	< 0·5	< 0·5	< 0·5
Triacetin	2	< 0·5	3	< 0·5	< 0·5	< 0·5
Tributyrin	3	1	3	0·5	0·5	0·5
Trilaurin	5	2	5	2	—	—
Triolein	5	2	3	1	0·5	2
Tripalmitin	> 10	> 10	> 10	> 10	—	—
Tristearin	> 10	> 10	> 10	> 10	—	—
Monoricinoleate	3	2	3	2	> 10	> 10
Arachis oil	5	4	4	3	0·5	3
Coconut oil	> 10	3	3	2	2	1
Olive oil	> 10	2	4	2	2	1

Action of PVP-I on Trichomonas

All movements of the test organism ceased within 4 min. exposure to 1/20 PVP-I 'Douche'; this was taken as the killing time.

Residual bactericidal activity of PVP-I after neutralization with sodium thiosulphate

No residual bactericidal activity could be demonstrated in the neutralized PVP-I sol. against the test organism. The diluted PVP-I sol. (1/100) killed the inoculum within 3 min.

Action of PVP-I on the skin flora

PVP-I as a cream and scrub was compared with chlorhexidine (1 %, w/w, cream), diethyl ether, ethanol, fucidin (1 %, w/w, cream), soap (white).

The flora of the hand was sampled, and the hand completely covered with an agent and left for 5 min. without drying. The hand was rinsed and the flora sampled again. The results (Table 4) show that none of the skin preparations sterilized the skin surface completely. Diethyl ether produced an increased count, presumably by its solvent action on fats, releasing bacteria from the deeper layers of the skin. The experiment was then extended to include an ether wash after skin disinfection to see if the deeper layers of the skin were sterile (Table 4). This showed that many bacteria in the deeper layers of the skin were unaffected by any of the antiseptics used.

Action of PVP-I sol. on the flora of the hand

The flora of a normal, unwashed hand was sampled by the velvet-pad technique; dipped in PVP-I sol. for 5 sec. and shaken; after a further 25 sec. the hand was rinsed and the flora re-sampled. This was repeated with the same hand on subsequent days, using 1/5 and 1/10 dilutions of PVP-I sol. (Plate 1*a*). In the concentrated solution and at both these dilutions PVP-I sol. sterilized the surface of the hand by this method of sampling except in one instance in which one colony of *Staph. albus* remained. The average volume used for a pair of hands per dip was 6–12 ml.

Table 4. *Effect of surgical preparing agents on the skin flora*

Skin treated with	Mean colony count of sampled area* and standard error		Percentage reduction	Mean colony count of sampled area after ether†
	Before treatment	After treatment		
Chlorhexidine 1 % cream (w/w)	82 ± 32	5 ± 1·44	94	25 ± 5
Diethyl ether	263 ± 113	—	—	581 ± 202
Ethanol	248 ± 145	5 ± 3·5	98	27 ± 22
Fucidin 1 % cream (w/w)	237 ± 211	106 ± 74	55	289 ± 109
PVP-I cream	145 ± 123	3 ± 4	98	9 ± 2
PVP-I scrub	223 ± 260	0·66 ± 1·19	99	48 ± 45
Soap (white)	139 ± 14	55 ± 34	53	131 ± 33

* *B. subtilis*, *Staph. albus* and *aureus*, and *Sarcina*.
† *Staph. albus*.

Duration of action of PVP-I

In surgical gloves

After the flora of the hand was sampled, the hand was scrubbed with PVP-I scrub for 5 min., rinsed, re-scrubbed for a further 5 min. and rinsed again. The skin was resampled and the hand placed in a surgical glove for 1 hr. After this period the glove was removed and the skin flora sampled. PVP-I scrub rendered the hand sterile on the surface; after 1 hr. in a surgical glove it remained sterile, as sampled by the velvet pad.

On exposed skin

A velvet pad swab of the hand was taken, the hand was then dipped in PVP-I sol. for 5 sec. After 1 min. the skin surface was re-swabbed and again at 30, 60, 90 and 120 min., the hand being used normally during this time. PVP-I sol. completely sterilized the skin surface and after 1 hr. it was still sterile; recolonization started after 1 hr.

The finger tips were deliberately infected with broth cultures of *Staph. albus*, *Esch. coli* and *Aerobacter aerogenes*, and then dabbed on a blood agar plate. The hand was then scrubbed in PVP-I scrub for two intervals of 5 min., rinsed and dried. The finger tips were then dabbed on to a blood agar plate, and again after 1 hr. This was repeated with chlorhexidine (0·5 % in 1 % stergene). The infected

finger tips were rendered sterile by PVP-I scrub, but with chlorhexidine in some cases some bacterial colonies remained. Recolonization of the finger tips started after 1 hr. (Plate 1*b*) with PVP-I scrub.

Bactericidal properties of residual PVP-I on the skin

The residual bactericidal properties of skin treated with PVP-I sol. were investigated as follows: The right hand was momentarily immersed in Lugol's iodine solution and the left hand in PVP-I sol. The hands were withdrawn and allowed to dry in air. After 1 hr., the right hand was placed into a beaker of 500 ml. of sterile water containing 1×10^7 *Staph. albus* per ml.; this was repeated with the left hand in another beaker. The hands were then agitated for 30 sec. to release any remaining antiseptic. After 2 min. viable counts were performed on the contents of the beakers. The hand previously dipped into PVP-I sol. killed the bacterial suspension in the beaker; the hand dipped into Lugol's iodine did not, a count of 5×10^6 organisms per ml. being recorded. A modification of the above experiment to determine if the skin surface became infected when immersed in bacterial suspensions was performed as follows: The flora of the finger tips was sampled before and after dipping them into PVP-I sol.; the residual PVP-I sol. was left on the skin for 1 hr. and the finger tips then dipped into suspensions of *Staph. albus*, *Esch. coli* and *A. aerogenes* (1×10^7 organisms per ml. in 15 ml. of distilled water); the finger tips were agitated for 30 sec., removed, and after 1 min. the skin surface was sampled; the bacterial suspensions were also sampled with a standard loop on nutrient agar, incubated for 12 hr. at 37° C.

Table 5. *A comparison of PVP-I with chlorhexidine–ether–meth. in skin preparation for ventricular tap and surgery*

	Mean colony count (PVP-I preparation)			Mean colony count (Chlorhexidine–ether–meth. preparation)	
Patient	Before	After	Patient	Before	After
1	41*	0	A	29*	3*
2	134*	0	B	36*	4*
3	10†	0	C	8*	0
4	51*	4*	D	39*	7*
5	196*	0	E	11*	0
6	32*	0	F	154*	11*

* *Staph. albus.* † Gram-positive rods.

One minute after dipping into the bacterial suspensions, the finger tips were sterile. Samples from all three bacterial suspensions were sterile except in one isolated instance in which four colonies of *A. aerogenes* were re-isolated.

Effect of PVP-I on the skin of the head

The heads of 6 children were shaved before the performance of intraventricular and other neurosurgical procedures. The skin was sampled. PVP-I scrub was then used, followed by a rinse, application of PVP-I sol. and re-sampling of the skin.

The results (Table 5) show that the treatment with PVP-I was more efficient than that with chlorhexidine. Repeated applications of PVP-I produced no sensitizing or other reactions.

In the course of these experiments with PVP-I, 2 subjects exhibited mild iodism, each being hypersensitive to other forms of iodine.

DISCUSSION

These results show that PVP-I has a rapid lethal action on vegetative cells of various bacteria and fungi *in vitro*. Many organisms, including bacteria resistant to various antibiotics, are killed in less than 1 min. When dense suspensions are used, certain organisms may survive for 5 min. but not longer. This rapid lethal effect is due to the liberation of free iodine from the complex, and is antagonized completely by the addition of sodium thiosulphate. Smooth surfaces such as glass and metal are also rapidly disinfected. The presence of organic material, such as agar, serum or whole blood, serves to delay but not to abolish the rapid bactericidal effect.

The action of PVP-I on spores is much weaker. Fungal spores are killed in $1\frac{1}{2}$ hr. but spores of clostridia and other bacilli survive for 17 hr. This is in contradiction to the report by Gershenfeld (1962) that PVP-I kills spores of *Bacillus subtilis*, *Clostridium tetani* and *Cl. welchii* in $2\frac{1}{2}$ hr.

In its action on the skin flora, PVP-I appears to be more effective than several other agents commonly used in preparing the skin for surgical procedures, in that it renders the skin surfaces sterile. This agrees with the findings of Lenhardt & Lachapelle (1961) and Joress (1962). Lowbury, Lilly & Bull (1963) found that PVP-I scrub was comparable with 2 % hexachlorophene soap. None of the agents used sterilizes the deeper layers of the skin, but PVP-I maintains a sterile skin surface for 1 hr. even in surgical gloves. Re-infection of a hand within 1 hr. after treatment with PVP-I is virtually impossible because of the residual PVP-I on the skin surface, which is enough to kill heavy bacterial suspensions in water. For general ward use PVP-I is acceptable. The prepared area is clearly defined by its colour but, unlike other formulations of iodine, it is readily washed out of skin and fabric by water. In our series, mild iodism occurred in two hypersensitive subjects. This is contrary to the assertion of Shelanski & Shelanski (1956) that PVP-I does not evoke this reaction.

SUMMARY

Polyvinyl-pyrrolidone-iodine complex (PVP-I) was shown to have a rapid disinfectant activity *in vitro* against vegetative bacteria and fungi and against vegetative *Trichomonas*. The presence of organic matter had a slight delaying effect on the killing time. Bacterial spores survived in PVP-I for 17 hr. and fungal spores for $1\frac{1}{2}$ hr. Used as an agent for pre-operative preparation of the skin, PVP-I was more efficient than others tested, rendering the skin surface sterile even when heavily contaminated, and keeping it so for 1 hr. None of the staining characteristics of iodine were noted since PVP-I was easily removed by water. Two individuals, known to be iodine sensitive, developed mild iodism after application of PVP-I.

The authors are indebted to the staff of the hospital for their co-operation; and to Berk Pharmaceuticals Limited for supplies of PVP-I, manufactured by them as Betadine (Povidone-iodine B.P.).

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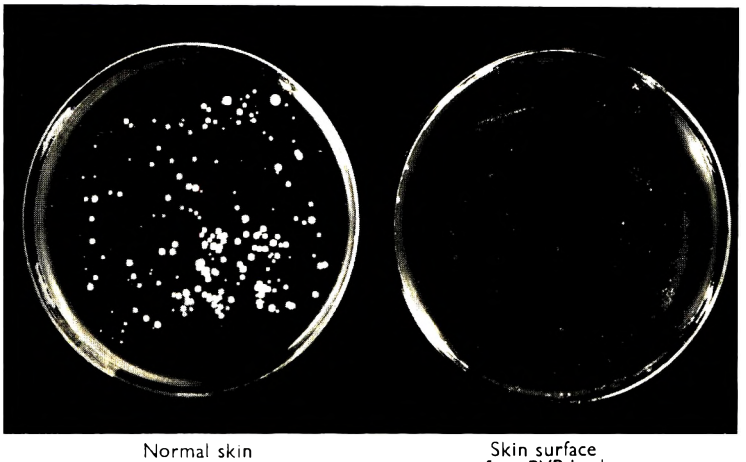
EXPLANATION OF PLATE 1

(*a*) Velvet pad samples of normal skin surface flora (*Staph. albus*) and skin surface after a 5 sec. dip in PVP-I sol.

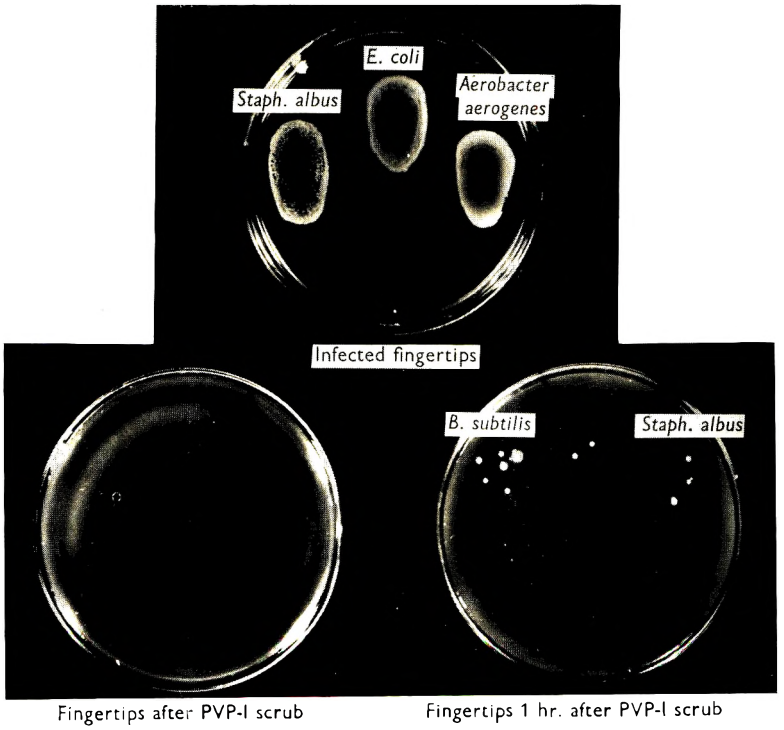
(*b*) Finger-print plates, show the sterilization of heavily infected finger tips by PVP-I scrub and re-colonization of the skin surface after 1 hr.



Normal skin surface flora

Skin surface after PVP-I sol.

(*a*)



Fingertips after PVP-I scrub

Fingertips 1 hr. after PVP-I scrub

(*b*)

J. Hyg., Camb. (1964), **62**, 519
Printed in Great Britain

Tests on self-disinfecting surfaces

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(*Received* 26 *June* 1964)

INTRODUCTION

It might help to reduce the spread of some infectious diseases if surfaces which are liable to bacterial contamination could be treated so as to make them able to kill organisms subsequently deposited on them. That this was so could be shown only by properly conducted field trials, but some information on the self-disinfecting properties of surfaces can be found from suitable laboratory tests. Some tests which have been used for this purpose are unsatisfactory because they do not simulate sufficiently closely the conditions under which the disinfectant has to act in practice. We have therefore attempted a critical evaluation of such tests, and have used a selected technique to assess the self-disinfecting properties of a variety of surfaces and treatments. Our methods are derived largely from those previously described by Klarmann, Wright & Shternov (1953), Lester & Dunklin (1955), and Morris & Darlow (1959). A recent discussion of techniques is given by Walter & Foris (1963).

Because of the small amounts of disinfectant available on a surface, no self-disinfecting surface is likely to be effective against massive contamination. Small drops of liquid contamination will dry rapidly, and dust settling on to a surface will be already dry. Thus, though it is useful to know the effect of the disinfectant on liquid inocula, the results with dry inocula are more generally relevant. These two types of inoculum give different results. If dry materials fall on to a dry, non-volatile, disinfectant layer, solid phase diffusion must take place before the disinfectant can reach the organisms, and this is a very slow process. Where a gaseous disinfectant such as formaldehyde is evolved (Hoffman, Kay & Feazel, 1959; Kingston, Lidwell & Noble, 1962) this limitation does not apply. With a liquid drop, some of the disinfectant will be dissolved and have a chance of reaching the organisms before the drop dries. Thus liquid inocula are often killed more readily than dry inocula. Also, care is needed in sampling since the disinfectant may be reactivated on wetting.

Dry pathogenic bacteria ordinarily occur in the environment associated with dried body fluids, and often on skin scales or textile fibres. Contamination is never likely to be with unprotected organisms, since the median equivalent diameter of airborne particles which carry bacteria is 10–14μ, indicating that there is much associated material (Noble, Lidwell & Kingston, 1963). In ordinary use surfaces

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rapidly become covered with small amounts of dust, and this will give still further protection to bacteria settling on them. For these reasons we thought that a fine cotton dust impregnated with organisms in broth suspension would be a suitable dry test inoculum.

For liquid inocula we used drops of broth culture, since contamination will ordinarily be with infected body fluids and so contain protein materials.

Bacteria can grow only at humidities above about 90% (Scott, 1957). Thus substances which merely inhibit growth will not reduce the numbers of bacteria on a dry surface. We have therefore used techniques designed to test specifically for bactericidal action. This limitation may not apply to fungi, and treatments and test methods used for them must therefore be critically examined before being used for bacteria.

MATERIALS

Organisms

Staphylococcus aureus (NCTC 9789). Phage type 80/81, isolated by Dr P. M. Rountree from a maternity infection.

Escherichia coli. Antigenic structure O.128:B.12:H.2, isolated from a case of infantile gastro-enteritis, and supplied by Dr Joan Taylor (her reference E56/59).

Before the start of the investigation a batch of ampoules was prepared for each organism, by distributing and drying a blood-broth culture.

Media

Broth. Oxoid nutrient broth No. 2.

Agar. Serum agar prepared from Oxoid blood agar base by the addition of 5% horse serum.

Diluent. Physiological saline with 5% broth added.

Resuspending fluid. Digest broth with 0·1% Tween 80 and one egg yolk in 500 ml. (Morris & Darlow, 1959). Tests for each disinfectant with three times the greatest amount which could be eluted showed that, when this fluid was used as described, there was unlikely to be killing during the elution of the bacteria or inhibition due to carry-over of disinfectant on to the plate.

Cotton dust

This was C4 white cotton flock, which is pure cellulose and corresponds to a bleached cotton fabric; it was kindly supplied by Messrs Hutchinson (Ramsey) Ltd.

Test surfaces

The blanket was cut into 1 in. squares, and the other materials into strips $\frac{5}{8} \times 1\frac{5}{8}$ in. (area 1 in.^{2}).

Blanket. 'Aertex' cellular cotton blanket.

Waxed wood. $\frac{1}{8}$ in. thick hardwood (Ramin) strip sprayed with dilute french polish and then coated with Traffic-Wax Paste (Messrs S. C. Johnson and Son Ltd.).

Painted wood. Similar strip painted with one coat of Dulux undercoat white 101 and two coats of Dulux gloss finish white 101 (Imperial Chemical Industries Ltd.).

Floor tile. A plastic floor tile kindly supplied by Marley Floorings Ltd.

Polythene. $\frac{1}{16}$ in. thick sheet (Tenaplas Ltd.).

Chipboard. $\frac{1}{8}$ in. thick strips cut from an old specimen tile. Since they were absorbent, they were coated with urea-formaldehyde resin for experiments with liquid inocula. (Chipboard is made from wood chips bonded with this plastic.) This material evolves formaldehyde (Kingston *et al.* 1962).

PFR (phenol-formaldehyde resin). $\frac{1}{16}$ in. thick laminated paper sheet.

Rubber. One of a series of experimental latex sheets kindly supplied by the British Rubber Producers' Research Association, selected because it produced a wide zone of inhibition on an agar plate seeded with *Staph. aureus*.

The waxed and painted wood, the floor tile and the blanket were used as vehicles for the disinfectant treatments. The floor tile was slightly absorbent, and thus provided an intermediate between the wood surfaces and the blanket. The other materials were tested without any disinfectant, because of the possibility that they might be inherently self-disinfecting, except for the Polythene which was included because it was thought to be unlikely to affect survival.

Disinfectants

Permachem (The Permachem Corporation Ltd.). Bis-tri-*n*-butyl tin oxide in association with various quaternary ammonium compounds. This was supplied in various formulations and the appropriate one used at the strength recommended by the manufacturers. This gave the following concentrations of the tin compound: wax, 0·1 %; blanket rinse, 0·2 g./kg. of dry blanket; dipping water for floor tile and painted wood, 0·004 %.

O-Syl (Lehn and Fink Products Corporation, U.S.A.). 12 % *o*-phenyl phenol in solution with potassium ricinoleate and glycols. Used at a 1/40 dilution. (There is a quite different British product of the same name.)

Savlon (Imperial Chemical Industries Ltd.). 0·3 % chlorhexidine digluconate and 3 % cetrimide. Used at a 1/40 dilution. In some experiments the effect of a moistening agent was investigated by adding 1 % glycerine to the solution used.

The blanket was given a final rinse in the disinfectant solutions, the other strips were dipped in them. The strips were then left in the open until dry.

METHODS

Inoculation of the surfaces

With liquid culture

A fresh ampoule of the dried organism was opened into broth. After overnight incubation a loopful of this culture was used to inoculate a 50 ml. bottle of broth. This broth after 18–24 hr. incubation provided the test inoculum. (All incubations were at 37° C.) Examination of the undried broth cultures by phase-contrast microscopy showed that the *Staph. aureus* culture consisted mainly of groups of two and four cocci, that of the *Esch. coli* of single bacilli. One drop (*c.* 0·02 ml.) from a standard dropper was allowed to fall on to each of the test strips; it was not spread deliberately, but on some of the materials it sometimes did so naturally. Two strips were sampled individually for each determination of survival.

With infected dust

A fresh ampoule of the dried organisms was opened into broth, incubated, and the culture flooded on to four agar plates. After overnight incubation the surface growth on these plates was suspended in 20 ml. of broth and mixed thoroughly with about 20 g. of cotton dust. The dust was then dried *in vacuo* over fused calcium chloride for 7–8 hr., and kept overnight in a chamber with saturated zinc nitrate solution to approximate to its humidity to 42 %. Before dispersal it was ground in a Waring blender and sieved in an exhaust-ventilated cabinet.

The survival during preparation was measured on one occasion and was found to be 17 % for *Staph. aureus* and 0·9 % for *Esch. coli*. Phase-contrast examination of the original suspension and of the suspension eluted from the dust showed that for *Staph. aureus* they both consisted of a wide range of aggregates of cocci, some of considerable size, whereas both of the *Esch. coli* suspensions consisted of single bacilli.

The prepared dust was blown into the top of a chamber 3 ft. × 3 ft. × 7½ ft. high, near a powerful fan. During this period the 60–80 test strips, which were set out on the floor of the chamber, were kept covered with a metal lid attached to the floor by hinges at one end. When the dust had been dispersed the fan was turned off, and after a short pause to allow violent air movement to die down, the cover was raised from outside the chamber. After allowing settling to take place for ¼ hr., the cover was lowered over the strips and a preliminary disinfection of the chamber carried out with ultra-violet light. (This method of disinfection is ineffective against bacteria screened by dust particles, but would kill any particles small enough to remain airborne for any length of time.)

There was considerable variation between the size of the inocula in different experiments; the counts were of the order of $0{\cdot}5 \times 10^6$ organisms per strip, corresponding to 1 mg. of dust. Within an experiment the variation of the counts was in excess of the theoretical minimum (Poisson), and gave a coefficient of variation of the order of 25 %. Accordingly, not fewer than five strips were sampled individually for each determination of survival.

It was possible to inoculate strips with the dust by sieving it manually on to them in a protective cabinet. The weight of dust was about 30-fold greater, but the uniformity, which was controlled by eye, was similar. Since the previous technique required special apparatus, it was thought that this might provide a more convenient alternative.

Estimation of survival

The strips were put into screw-cap 1 oz. bottles ('universals') containing 10 ml. of egg yolk Tween broth and shaken vigorously. The blanket strips were squeezed out and re-wetted several times. Appropriate dilutions were made in broth-saline and five or ten drops from a standard dropper were inoculated on to the surface of well-dried serum agar plates. Colonies were counted after overnight incubation at 37° C. The drops of inoculum were not spread on the medium.

Storage at constant humidity

The inoculated strips were stored at a constant humidity of 42 % and in a very dim light. Those with the dust inoculum were placed in the humidity chambers as soon as the ultra-violet disinfection of the settling room was completed. Owing to the amount of work involved in tests with the drop inocula, these strips were left at laboratory humidity until the 2 hr. sample had been taken.

The strips treated with different disinfectants were kept in different chambers, but there was no evidence that any except the formaldehyde-evolving compounds gave off toxic vapours. With the surfaces which evolved formaldehyde, organisms on control strips in the same chamber were killed and the rate of kill on the test strips was increased. Survival of these surfaces was therefore measured in the open in a well-ventilated room in a dim light. A continuous record was kept of the humidity in this room, and during the experiments it did not differ greatly from 42 %.

The constant-humidity chambers were trays 15 in. × 20 in. × 5 in. deep closed with heavy sheets of glass lying on sponge rubber gaskets. The humidity was controlled with saturated zinc nitrate solution containing excess solid; this is in equilibrium with a relative humidity of 42 % at 20° C. (O'Brien, 1948). The solution had a total surface area of 110 in.^{2}, and the specimens were kept on racks 1½ in. above the surface. Tests showed that under these conditions adequate humidity control was likely to have been achieved, the criticisms of Martin (1962) applying mainly where large masses of absorbent materials are present.

The choice of humidity is important as it affects the natural death rate of the organisms, the rate at which the disinfectant can diffuse to them and the bactericidal activity of the disinfectant. Indoor measurements taken day and night throughout the year do not seem to be available. Daytime readings for the winter months in offices in Newcastle-upon-Tyne and in London (Lidwell & Williams, 1961) gave limits for a series of interquartile ranges as 31 and 62 %. Hourly outdoor wet and dry bulb readings taken for 8 years at Birmingham airport, and kindly supplied by the Meteorological Office, when converted to internal humidity values by an expression inferred from the previous data (internal R.H. = external millibars × 2·8 +26) suggested an interquartile range of 46–60 %. Thus the majority of humidity measurements in centrally heated buildings in England would probably lie between 35 and 60 %. Since zinc nitrate was readily available we adopted 42 % as the standard.

Zones of inhibition

Serum agar plates were flooded with a 1/100 dilution of an overnight broth culture of the organism. As soon as surface moisture had been absorbed, the inhibitory material was placed on the surface of the agar and the plates placed at once in the incubator, stacked not more than two deep. The effect of the liquid disinfectants was tested by using squares of blanket appropriately treated and dried, of the other substances by using strips of the test material itself. The zones of inhibition were examined after 18–20 hr. incubation and are reported as the width of the zone from the edge of the strip.

We think that this method of testing for self-disinfecting activity may be most misleading. However, some tests were done with it so that the results could be compared with those obtained by the other methods.

RESULTS

Control surfaces

Table 1 gives a summary of the percentage survivals found with dust inocula, Table 2 of those with liquid inocula. Preliminary inspection showed no significant difference between the survivals of drop inocula on Polythene, waxed wood, painted wood and floor tile, and these have accordingly been pooled. The values for the mid-point and the scatter of the different distributions, expressed as the median and the interdecile range, were found by plotting cumulative distributions on prob-

Table 1. *Percentage survival of dust inocula on untreated surfaces*

Interval from inoculation	Floor tile		Blanket	
	Median	Interdecile range	Median	Interdecile range
		Staph. aureus		
24 hr.	32	10–54	73	63–104
4 days	5·4	0·3–16	23	8–37
		Esch. coli		
24 hr.	27	7–47	25	18–33
4 days	15	4–25	13	2–23

The median is the point above and below which equal numbers of determinations lie. The interdecile range is the range within which 80 % of the determinations lie, i.e. the range excluding the highest and lowest tenths.

Number of determinations for each percentage survival: *Staph. aureus*: floor tile, 6; blanket, 5; *Esch. coli*: floor tile, 7; blanket, 4.

Table 2. *Percentage survival of drop inocula on untreated surfaces*

Interval from inoculation	Other than blanket*		Blanket	
	Median	Interdecile range	Median	Interdecile range
		Staph. aureus		
0 hr.	100	82–117	106	56–156
2 hr.	126	87–165	58	11–140
5 hr.	157	101–213	32	0·6–130
24 hr.	157	101–213	23	4·3–120
5 days	141	68–214	3·9	0·3–11
		Esch. coli		
0 hr.	100	85–115	62	37–87
2 hr.	131	37–190	0·63	0·07–5·6
5 hr.	55	13–150	0·089	0·04–0·3
24 hr.	18	0·2–66	0·040	0·01–0·2
5 days	3·4	0·1–23	0·0072	0·003–0·02

Number of determinations for each percentage survival: blanket, 11; other surfaces, about 35.

* Polythene, waxed wood, painted wood, floor tile.

ability paper. For the dust-borne organisms the numbers of determinations were small. However for *Staph. aureus* there were, from another series, nine determinations of survival at 24 hr. on floor tile. When these were included the distribution was not significantly changed. Thus the data presented are probably representative.

The results show that determinations of survival carried out under conditions practicable for routine tests give results which are more variable than is often assumed. The survivals tended to be consistently high or low for any one run. This suggests that it would be better to compare the survival on a test surface with the survival on its individual control. However, in the present series of experiments it was found that the reproducibility of duplicate estimations found in different experiments was similar whether comparison was made with the individual or the pooled values. In general, this variability indicates that a number of replicate determinations are needed before it is safe to say that different strains or different conditions give different survivals.

Time of drying

The drops ordinarily dried between the 2 hr. and the 5 hr. sample; the time of drying on the blanket was not determinable.

Table 3. *Survival of dust inocula on treated surfaces as percentage of survival on untreated surfaces*

		Staph. aureus				*Esch. coli*			
Treatment	Interval from inoculation	Floor tile		Blanket		Floor tile		Blanket	
Permachem	1 day	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days		+ + +		+ + +	+ + +	+ + +	+ + +	+ + +
Savlon	1 day	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
Savlon + glycerine	1 day	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days	+ +	+ + +	+ + +	+ + +	+ +	+ +	+ + +	+ + +
O-Syl	1 day	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	4 days		+ +		+ + +	+ +	+ + +	+ +	+ + +

		Staph. aureus						*Esch. coli*					
		Rubber		PFR		Chipboard		Rubber		PFR		Chipboard	
Untreated miscellaneous surfaces	1 day	+ + +	+ + +	+ + +	+ +	−	+	+ + +		+ + +		+	−
	4 days	+ + +	+ + +	+ + +	+ +		+	+ + +		+ +			−

+ + +, Survival greater than 50 % of control; + +, survival between 50 and 10 % of control; +, survival between 10 and 1 % of control; −, survival less than 1 % of control. Each of these represents the results of a separate experimental determination. PFR, Phenol-formaldehyde resin.

Disinfectant surfaces

The results for dust inocula are given in Table 3, for liquid inocula in Table 4. They are given in terms of the median values set out in Tables 1 and 2. Since only big differences were considered significant the results have been grouped and tabulated in the following manner: + + +, survival greater than 50 % of control;

Table 4. *Survival of drop inocula on treated surfaces as percentage of survival on untreated surfaces*

Treatment	Interval from inoculation	*Staph. aureus*									*Esch. coli*									
		Waxed wood		Painted wood		Floor tile		Blanket			Waxed wood		Painted wood		Floor tile		Blanket			
Permachem	2 hr.	+++	+++	+++	+++	+++	+++	+++	+++	+	++	+++	+++	+++	++	+++	+++	+++	+++	
	5 hr.	+++	+++	+++	+++	++	++	+++	+	−	++	++	++	++	++	+++	+++	+++	+++	
	1 day	+++	+++	+++	+++	++	++	+++	+	−	++	+	++	+++	+++	+++	++	+++	+++	
	5 days	++	++	++	+++	++	+	+	+	−	++	+++	+	+	++	+++	+++	+++	+++	
Savlon	2 hr.	+++		+++		+++		−	−	−	+++		+++		++		+++	+++	⩽ +	
	hr	+++		+++		++		−	−	−	++		+		++		++	⩽ +	−	
	1 day	+++		+++		++		−	−	−	+++		++		+++		++	⩽ +	+	
	5 days	++		+++		++		−	−	−	+++		+++		+++		⩽ ++	⩽ ++	++	
Savlon+glycerine	2 hr.	+++	+++	+++	+++	+++	+++		++		+++	+++	+++	+++	+	+++		++		
	5 hr.	+++	+++	+++	+++	−	++		+		+++	+++	+++	+++	++	+++		++		
	1 day	+++	+++	+++	+++	+	++		+		+	+++	+	+++	−	+++		++		
	5 days	+++	+++	+++	+++	−	+		−		++	+++	++	+++	−	+++		++		
O-Syl	2 hr.	+++	+++	+++	+++	−	++		−		+	+++	+	+++	⩽ +	+++		⩽ +		
	5 hr.		++		++		−		−			++		++		+		⩽ +		
	1 day	+++	++	+++	+++	+	+		−		−	++	−	+	−	++		⩽ +		
	5 days	+++	++	++	+++	+	+		−		−	+++	−	−	−	−		⩽ ++		

Treatment	Interval from inoculation	*Staph. aureus*				*Esch. coli*			
		Rubber		PFR	Chipboard	Rubber		PFR	Chipboard
Untreated miscellaneous surfaces	2 hr.	+++	+++	+++	++	++	++	+++	++
	5 hr.	+++	+++	+++	+	+	++	+	−
	1 day	+++	++	+++	−	+	++	++	−
	5 days	+++	++	+++	−	++	++	+	−

+++, Survival greater than 50% of control; ++, survival between 50 and 10% of control; +, survival between 10 and 1% of control; −, survival less than 1% of control. ⩽ Equal to or less than the survival shown; no colonies were found at the dilutions tested, and the value given is that which would have resulted from there being three. Each of these represents the result of a separate experimental determination.

+ +, survival between 50 and 10 % of control; +, survival between 10 and 1 % of control; −, survival less than 1 % of control. Thus + + + implies that survival was similar on the control and the test surfaces, but + and − indicate some action of the disinfectant. In assessing the value of the results it must be remembered that to be useful, a reduction must be at least comparable with that brought about by cleaning.

Dust inocula

The results with dust inocula are clear-cut; none of the surfaces showed any useful lethal effect except the chipboard, which evolves formaldehyde. A more extensive investigation of the ability of surfaces which evolve formaldehyde to kill organisms present in dust has been reported in a previous paper (Kingston *et al.* 1962). The addition of glycerine to the Savlon might have facilitated the diffusion of the disinfectant into the bacteria by remaining liquid, but no increased kill was found.

Liquid drop inocula

With these inocula there is more chance of the disinfectant reaching the organism, but the degree of kill will be dependent on the amount of disinfectant available. On the wood surfaces there was no indication of useful kill with *Staph. aureus* suspensions, and on *Esch. coli* only O-Syl had a marked, though somewhat irregular, effect. Tests had shown that *Esch. coli* survived less well if spread over the surface, and since the surface-active agents sometimes caused the drop to spread, some of the slight kill observed with this organism may be due to an effect of spreading.

In contrast to the wood surfaces, which even if wetted retained only a thin film of disinfectant, the floor tile absorbed an appreciable amount, and substantial kills were sometimes found, generally when the drop had spread. (A drop will come into contact with more of the disinfectant on spreading.) Some examples are given in Table 5. A drop was said to have spread if the normal drop outline was not present. In such cases the drop had usually spread across the width of the strip. The effects were much greater than those found when drops were spread in the absence of disinfectant.

The blanket strips absorbed the disinfectant and allowed the drops to spread, and as expected good kills were found (Table 4). The results for *Esch. coli* were rather irregular, in part because this organism survived very poorly when applied to blanket in a drop of broth (Table 1), so that extra kill due to disinfectants was difficult to show. In addition, quaternary disinfectants, which are part of the formulation of Savlon and of Permachem, are less effective against intestinal organisms than against pyogenic cocci. Permachem gave rather irregular results with both organisms, probably because it sometimes prevented the drop of inoculum from being absorbed readily.

The chipboard gave good kills with drop inocula as well as with dust inocula, but the rubber and PFR showed no significant effect.

These results can be summarized by saying that under the conditions of test used here, liquid inocula were killed where there was enough disinfectant absorbed,

particularly if the ratio of disinfectant to inoculum was increased by the drop spreading. Dust inocula were not killed, except by those surfaces which evolved formaldehyde, and these surfaces also killed drop inocula. The addition of glycerine to the Savlon solution did not potentiate kill.

Table 5. *Effect of spreading on the survival on treated floor tile; survival as percentage of inoculum*

Organism	Treatment	Time from inoculation	Percentage survival	
			Drop	Spread*
Staph. aureus	Savlon + glycerine	2 hr.	84	8
		5 hr.	61	⩽ 5
		1 day	31	*c.* 0·004
	O-Syl	2 hr.	61	4
		5 hr.	1	⩽ 0·3
		1 day	6	0·3
		5 days	6	⩽ 0·003
Esch. coli	Savlon	2 hr.	45	1
		5 hr.	17	0·3
		1 day	9	⩽ 0·4
		5 days	2	*c.* 0·008
	Savlon + glycerine	5 hr.	100	1
		1 day	73	0·02
		5 days	25	0·05
	O-Syl	1 day	2	⩽ 0·03
		5 days	*c.* 0·03	*c.* 0·001

⩽ Equal to or less than the survival shown; no colonies were found at the dilutions tested, and the value given is that which would have resulted from there being three.

* The spread drops are those which had spread naturally. The area over which this had occurred was variable, but generally of the order of $\frac{1}{3}$ in.^{2}.

Table 6. *Width of zone of inhibition*

Material	*Staph. aureus*	*Esch. coli*
Blanket	0	0
Blanket (Permachem rinse)	3	0
Blanket (Savlon rinse)	2	0
Blanket (O-Syl rinse)	Trace	Trace
Rubber	6	3
PFR	0	0
Chipboard	3	0

The zone width is given in mm. and is the width from the edge of the strip. The lawns were prepared by flooding serum agar plates with a 1/100 dilution of overnight broth cultures.

Zones of inhibition

The results of this test are given in Table 6. The width of the zone bore no relation to the killing effect as judged by other tests. Thus rubber, which gave no useful kill in the other tests, gave good zones of inhibition on lawns of both organisms. Chipboard, which killed both organisms, gave a zone with *Staph. aureus* only. Tests with other surfaces which evolved formaldehyde gave similar zones, thus confirming

that they were due to formaldehyde and not to other constituents of the chipboard. Blanket treated with O-Syl, which killed both organisms in drop inocula but neither in dust inocula, gave very small zones with both organisms.

The inhibition zone test does not distinguish between bactericidal and bacteriostatic action, and there are two other features which make it liable to indicate unjustifiably that materials tested might have a self-disinfecting action: the bacteria are in good liquid contact with the disinfectant and they may be actively multiplying. Conversely, if the disinfectant were diluted out by the comparatively large bulk of the agar, useful materials might be missed.

The widths of the zones found with different specimen sheets of rubber suggested that the constituent responsible was zinc dimethyl-dithio-carbamate or its homologues.

DISCUSSION

Many authors have found that liquid inocula can be killed by surfaces coated with dried disinfectants; for example, Klarmann *et al.* (1953) who treated a number of surfaces with a variety of disinfectants and then spread loopfuls of culture over them, and Rountree (1946) who impregnated blankets with cetyl pyridinium bromide and sprayed them with streptococci. Both these techniques increase the amount of disinfectant available to the organisms by dispersing them over a large area, and this may explain the high degree of kill these authors found. It is also probable that the elution technique of Morris & Darlow (1959) is more satisfactory than the methods of culturing used by many of the earlier workers, since it is easier to make sure that there has been no kill during sampling, or inhibition of growth due to carry-over of disinfectant. Our results suggest that many surfaces do not absorb enough disinfectant for useful kills to occur, and that the amount of kill is never very large unless the drop spreads.

With dust inocula the only surfaces we found to show any useful kill were those which emitted formaldehyde. Morris & Darlow (1959) tested paints containing disinfectants with organisms settled on to them in 10–15μ diameter particles. They found that, particularly with cetavlon in the paint, good kills were obtained at 70% R.H., but that unless the organisms were sprayed in glycerol-buffer solutions kills were very poor at 40% humidity. Rubbo, Stratford & Dixson (1960) found that none of the treatments they tried, which included Permachem and quaternary ammonium compounds but not O-Syl, reduced the level of contamination on blankets in use in the wards. Finegold *et al.* (1962) found no self-disinfecting activity when floors were treated with quaternary ammonium compounds, Permachem, or Amphyl (a disinfectant similar to O-Syl). Our results support these findings. However, Lester & Dunklin (1955) were able to demonstrate some kill by surfaces treated with O-Syl at humidities down to 33% and even with materials as poorly absorbent as glass. Their test contamination was spray-dried streptococci contained in particles less than 5μ in diameter and, more relevantly, sieved room dust. The reduction in counts they found was not large, but our slightly different test system did not give any useful effect at all. They also showed a large reduction under field conditions, but at humidities rather higher than are usual in England (Dunklin & Lester, 1959).

Claims have been made for rubber as a self-disinfecting surface (e.g. Nopitsch & Möbus, 1958; Dempster, Davy & Swanson, 1959), based chiefly on the ability of rubber either to produce a zone of inhibition on a seeded plate or to kill bacteria maintained in prolonged liquid contact with it. We believe such tests to be misleading.

The self-disinfecting surfaces which seem to us to show the greatest promise are those which slowly evolve formaldehyde. Hoffman *et al.* (1959) give details of a variety of compounds tried as formaldehyde donors, and conclude that paraformaldehyde is the most satisfactory. They also point out that these surfaces will kill spores as well as vegetative forms. Morris & Darlow (1959) found that paints containing paraformaldehyde killed less rapidly than those containing cetavlon. However, the lethal action of surfaces which evolve formaldehyde continues satisfactorily even after drops of broth culture have dried on them, and they are the only surfaces we have found to have a useful effect on dust-borne organisms. The data given by Kingston *et al.* (1962) show that such surfaces kill dust-borne organisms even after several months storage and after being washed.

It is interesting that Caplan (1962), examining the bacterial contamination of blankets after use in hospital wards, found lower levels on those which had previously been sterilized with formaldehyde. Dr V. G. Alder of Bristol (personal communication) has confirmed that blankets which have been sterilized with formaldehyde have self-disinfecting activity. Specially treated fabrics which evolve formaldehyde can cause dermatitis (Marcussen, 1959), though the workers at Bristol have not been troubled with this.

We think the tests we have used simulate the conditions under which self-disinfecting surfaces would need to act in practice, though we may have judged the level of contamination wrongly. However, even if such tests show a treatment to be promising, it does not necessarily follow that it will be useful. For example, cleaning may well be more effective than disinfection in reducing environmental contamination. Finegold *et al.* (1962, 1963) found that the reduction in environmental contamination brought about by certain disinfectant treatments appeared to be due to the cleaning involved in their use. They also found that the reduction in contamination did not bring about a reduction in morbidity.

SUMMARY

A variety of surfaces was tested for self-disinfecting action against *Staph. aureus* and *Esch. coli* at a humidity of 42 %. Surfaces which had been treated with disinfectants sometimes reduced the survival when the organisms were applied subsequently in drops of broth, but the reduction was very small unless the surface was able to absorb appreciable amounts of disinfectant and the drop of inoculum spread. When the inoculum was applied dried in cotton dust, only surfaces which evolved formaldehyde showed any activity against the bacteria. These surfaces also reduced the survival of bacteria in liquid inocula.

We would like to record our thanks to Dr O. M. Lidwell for much helpful advice.

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