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A simplified method for the detection of rubella-specific IgM employing sucrose density fractionation and 2-mercaptoethanol

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SUMMARY

A simplified method for the detection of rubella-specific IgM in sera was developed involving HAI tests on only one fraction from a sucrose density gradient with and without treatment with 2-mercaptoethanol (2 ME). Slight trailing of IgG into the IgM fraction was shown to occur in patients with high titres of both IgG and IgM, but this could be detected by the 2ME treatment and did not affect the results.

By this test, rubella-specific IgM was found in all of 54 patients with a rash and a rise of rubella HAI antibodies. When the antigen and serum were incubated for 1 hr. before adding the RBC, specific IgM was found in all 51 sera taken between 5 and 40 days after onset, and in about half the sera taken between 42 and 77 days, but in none of 16 people known to have possessed rubella antibodies at least a year previously. When antigen and serum were incubated for 18 hr. before adding the RBC, specific IgM was found in all of 21 sera taken between 2 and 49 days after onset, and in 6 of 11 sera taken between 50 and 117 days, but not in 9 people known to have possessed rubella antibodies at least a year before. The method has been found to be very useful in detecting recent rubella among patients in early pregnancy.

INTRODUCTION

A common problem in virology is the diagnosis of patients in early pregnancy who are not seen until more than a week after a rash or more than three weeks after contact with a case of rubella. Conventional laboratory tests may not help to decide whether the patient has had rubella during her pregnancy. The presence of rubella-specific IgM immunoglobulin seems to indicate recent infection (Vesikari & Vaheri, 1968; Best, Banatvala & Watson, 1969; Desmyter, South & Rawls, 1971; Ogra *et al.* 1971; Cradock-Watson, Bourne & Vandervelde, 1972; Haire & Hadden, 1972; Field & Murphy, 1972; Forghani, Schmidt & Lennette, 1973). This has been detected by 2-mercaptoethanol (2ME) treatment of whole serum (Banatvala *et al.* 1967), immunofluorescence (Baublis & Brown, 1968; Cohen, Ducharme, Carpenter & Deibel, 1968), separation of immunoglobulins in sucrose density gradients followed by rubella haemagglutination inhibition (HAI) (Vesikari & Vaheri, 1968) or radioimmunodiffusion (Ogra *et al.* 1971), separation in a Sephadex column (Gupta, Peterson, Stout & Murphy, 1971) or agarose (Bürgin-Wolff, Hernandez & Just, 1971) followed by HAI.

Each of these techniques has disadvantages. With sucrose gradient separation there may be 'trailing' of IgG into the IgM fraction, and the technique is laborious. We tried to overcome the first difficulty by doing HAI tests with and without treatment of the individual fractions with 2ME, which disrupts IgM; we tried to reduce the labour by collecting and testing only one fraction after a careful calibration of the system. This simplified test was done on sera from 54 proved cases of acquired rubella and 16 normal adults who were known to have possessed rubella antibodies at least a year before. Most workers have used normal adults as control subjects, but it is possible that these people may be having subclinical rubella. After our method had been developed, Field & Murphy (1972) described a similar technique.

MATERIALS AND METHODS

Sera

These were collected from:

(1) Fifty-four patients with clinical rubella confirmed by a four-fold or greater rise of titre of rubella HAI antibodies. Six were children aged 7–15 years and 48 were adults of whom 19 were pregnant. Two sera were collected from each of 20 patients and three from each of 34. Sera from three of these patients were used for the calibration tests (see below). The date of onset of the rash was recorded as precisely as possible, but it was realized that in a few patients this information may have been inaccurate.

(2) Six patients whose serum contained rheumatoid factor as well as rubella HAI antibody.

(3) Sixteen laboratory staff who were known to have possessed rubella HAI antibodies at least 1 year previously. One of these sera was used in the calibration tests.

All sera were stored at $-20°$ C. and examined within 18 months of collection without inactivation.

Preliminary treatment of sera

Before gradient separation, 0·25 ml. of serum was mixed with 0·25 ml. of dextrose–gelatin–veronal buffer (DGV) and 0·05 ml. of 50% suspension of day-old chick RBC, incubated for 1 hr. at 4° C. and centrifuged to remove the RBC. A standard serum containing rubella-specific IgM was included in every run.

Sucrose density centrifugation

Sucrose solutions of 12·5, 18·75, 25·0, 31·0 and 37·5% (w/v) were made in DGV and kept at 4° C. Discontinuous gradients were made by layering 0·7 ml. amounts of each solution into 5 ml. MSE polycarbonate ultracentrifuge tubes. Immediately, 0·4 ml. of treated serum was placed on top. The tubes were then centrifuged in a 6×5 titanium swing-out rotor in an MSE superspeed 75 ultra-centrifuge for 18 hr. at 4–5° C. at 100,000 g with the brake off. Fractions were collected at once with an MSE tube piercer. Twelve fractions each of 25 drops

(0·3 ml.) were collected and used for the early calibrations. In later routine estimations only the second 50 drops (pooled fractions 3 and 4) were collected.

Rubella HAI tests

The fractions were divided into two equal volumes; to one was added 0·05 ml. of 0·5 M 2ME (made up fresh every three weeks and stored at 4° C.), and to the other 0·05 ml. of DGV. Both were incubated in a water bath at 37° C. for 1 hr. and the samples were then titrated for rubella HAI antibodies in a microtitre test with 4HA units of antigen, 0·16 % day-old chick RBC and DGV. The antigen and serum dilutions were allowed to react for 1 hr. (short method) or for 18 hr. (long method). The tests were read after the RBC had settled for 3 hr.

HAI tests on whole sera were done in the same way, except that non-specific inhibitors were first removed with kaolin, 2ME was not used, and short fixation was always used.

Detection of IgA, IgG and IgM immunoglobulins

The fractions were tested by immunodiffusion in an agarose gel against anti-IgA, -IgG or -IgM sera (Hyland). The plates were examined at 1, 2 and 3 days and the amount of precipitate roughly estimated by eye and graded as ±, +, + + or + + +.

RESULTS

Calibration of centrifugation technique

A detailed examination was made of sera from three patients with clinical rubella and a rise in HAI antibodies, and from one normal subject. After separation in sucrose density gradients, all 12 fractions were examined for rubella HAI antibodies by the short method with and without treatment of the fractions with 2ME, and each fraction was tested for total IgA, IgG and IgM by immunodiffusion. The immunodiffusion test was read after three days in order to detect very small amounts of immunoglobulins which might have trailed into neighbouring fractions.

In patient A (Fig. 1) one day after the onset of the rash there was a low titre of antibody in fractions 3 and 4, which was completely removed by 2ME. These two fractions contained maximum IgM in the immunodiffusion tests and it was thought that all the rubella antibody was IgM. There was a low titre of antibody in fractions 6, 7 and 8 which was unaffected by 2ME, and immunodiffusion tests showed that these fractions contained IgA and IgG, but no IgM. Fractions 9, 10 and 11 had higher HAI titres than the other fractions, which were unaffected by 2ME. These fractions were thought to contain lipoprotein non-specific inhibitor mixed with a little IgG.

In patient A 11 days after the onset of the rash (Fig. 2) the titres of fractions 3, 4 and 5 were reduced by 2ME, showing that all three contained IgM. However, in fractions 4 and 5 antibody was not removed completely, showing that some IgG had been brought down into these two fractions. A similar result was seen with another patient B 5 days after onset, and with patient C 11 days after onset (Fig. 3). Patient C had a higher titre of rubella antibodies in both the IgM and

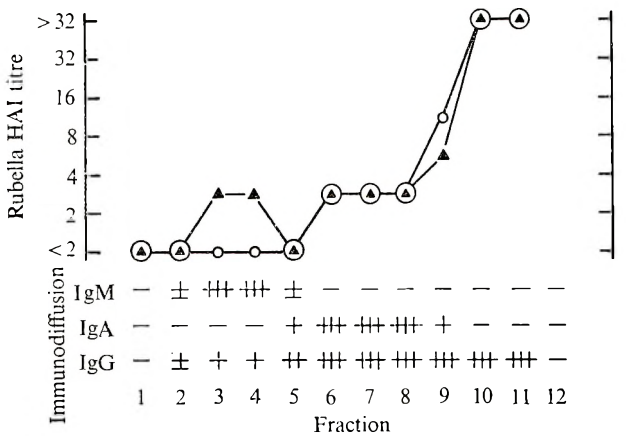

Fig. 1. Immunodiffusion against anti-IgM, IgA and IgG and rubella HAI on sucrose gradient fractions of serum from patient A taken 1 day after rash. ▲—▲ = without 2ME treatment; ○—○ = with 2ME treatment; − = no precipitate; ±, +, + +, + + + = amount of precipitate estimated by eye.

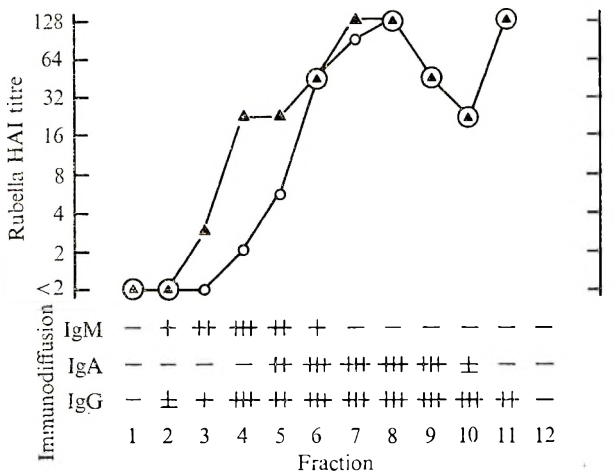

Fig. 2. Immunodiffusion against anti-IgM, IgA and IgG and rubella HAI of sucrose gradient fractions of serum taken from patient A 11 days after rash. ▲—▲ = without 2ME; ○—○ = with 2ME; − = no precipitate; ±, +, + +, + + + = amount of precipitate estimated by eye.

IgG fractions. However, when subject D (Fig. 4), who was known to have possessed rubella antibodies at least seven years before, was tested, all the rubella antibodies were in fractions 6 and upwards and all were unaffected by 2ME. The rubella antibodies were localized in the IgG fractions 6, 7 and 8 with no spread into either the lower IgM fractions or the higher non-specific inhibitor fractions. A similar result was found with serum from another normal subject.

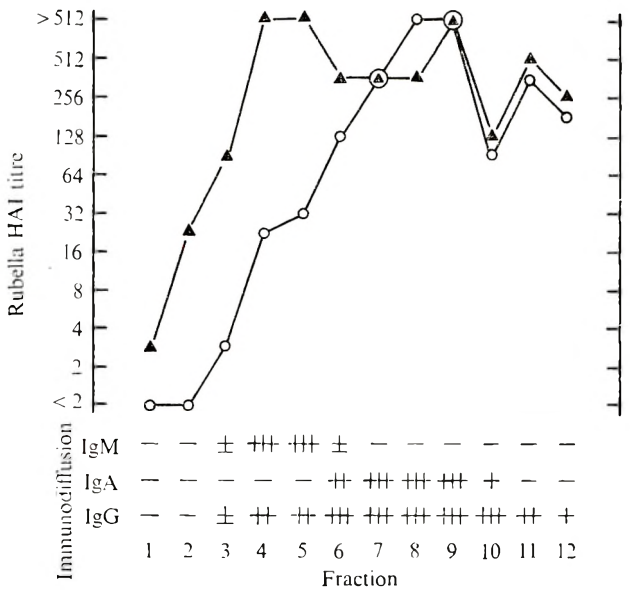

Fig. 3. Immunodiffusion against anti-IgM, IgA and IgG and rubella HAI of sucrose gradient fractions of serum from patient C taken 11 days after rash. ▲—▲ = without 2ME; ○—○ = with 2ME; − = no precipitate; ±, +, + +, + + + = amount of precipitate estimated by eye.

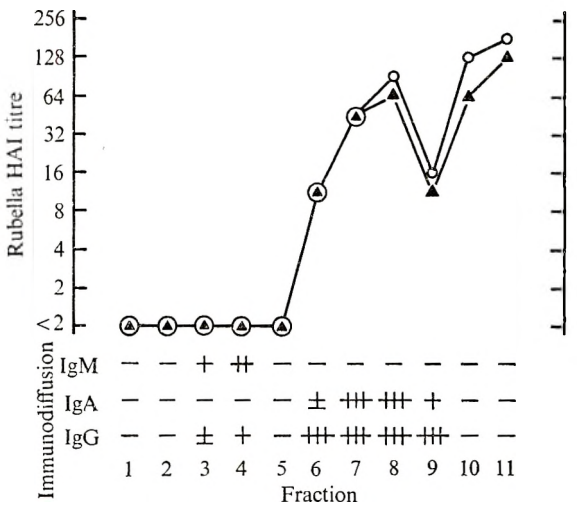

Fig. 4. Immunodiffusion against anti-IgM, IgA and IgG and rubella HAI of sucrose gradient fractions of serum from subject D. ▲—▲ = without 2ME; ○—○ = with 2ME; − = no precipitate; ±, +, + +, + + + = precipitate estimated by eye.

Identification of the residue

To characterize the residual inhibitor after 2ME treatment of fractions 3 and 4, these fractions pooled from a serum containing a high titre of IgM were treated with and without 2ME. Each lot was then divided into four parts and treated with an equal volume of phosphate-buffered saline, or of antiserum to either IgA,

IgG or IgM. After incubation for 1 hr. at 37° C., these mixtures were tested for rubella HAI antibody. In the part without antiserum there was residual activity after treatment with 2ME, but after mixing with anti-IgG this disappeared. Anti-IgM reduced the titre before treatment with 2ME but not after. Anti-IgA did not affect the titres. From this it was concluded that the residue was IgG.

Simplified test for rubella-specific IgM

From these results it was decided to collect the second 50 drops from the sucrose gradient (fractions 3 and 4 pooled) and not other fractions. Rubella HAI tests were done with and without treatment with 2ME. Any inhibition which was reduced at least 3-fold by the 2ME was considered to represent rubella-specific IgM. Fractions were not routinely tested by immunodiffusion.

All sera were tested without preliminary inactivation, although it was shown on one occasion that inactivation for 30 min. at 56° C. did not affect the IgM titre.

Length of incubation

After most of the tests described below had been done by the short method, it was suggested that incubation of the antigen and serum overnight at 4° C. before adding the RBC might make the test more sensitive (Pattison & Mace, 1973). In a comparison of the long and short methods on 20 sera containing IgM, the long method resulted in 2- to 8-fold higher titres (mean: 4-fold).

Reproducibility

Repeated tests on sera containing IgM showed only a 4-fold variation in 13 tests and a 2-fold variation in 8 tests by the short method, and a 4-fold variation in 7 tests and a 3-fold variation in 14 tests by the long method.

Rubella-specific IgM titres in patients with rubella

Including the 3 patients on whom the calibration tests were done, rubella-specific IgM was measured in a total of 54 patients. All had had a rash and shown a 4-fold or greater rise in rubella HAI on whole serum. The IgM test was done on 1 serum from each of 15 patients, 2 sera from each of 25 patients and 3 sera from each of 14 patients. Sera taken early in the illness, where the HAI titre of whole serum was less than 24, were not usually tested for IgM. Because of technical difficulties, fraction 5 was tested instead of fractions 3 and 4 in six cases. Titres were similar to those found with other sera taken at the same time, and so have been included in the results. In 10 patients there was some doubt as to whether the date of onset of the rash was accurate; however, since the IgM titres were similar to those of other patients, these 10 have been included. Most of the sera were tested by the short method, but after it was shown that overnight incubation gave higher titres, sera taken between 42 and 74 days after onset which were negative by the short method were repeated by the long method. A few sera were tested only by the long method.

Rubella-specific IgM was found at some time in all 54 patients. The titres reached a peak 7 to 12 days after the rash and then gradually declined (Fig. 5).

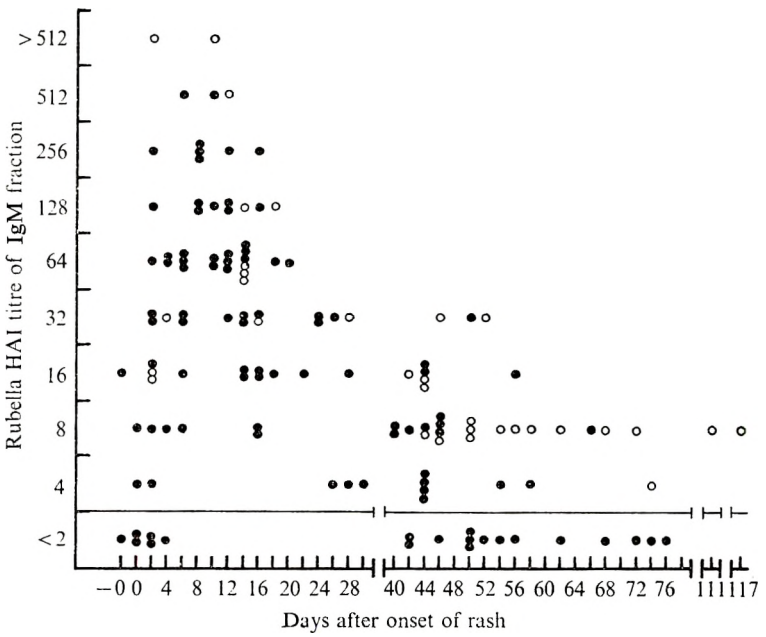

Fig. 5. Rubella HAI titres of IgM fractions of sera from 54 patients with proved rubella. ● = short method; ○ = long method. Figure includes 26 sera tested by both methods.

By the short method, specific IgM was found in all 51 sera taken between 5 and 40 days after the rash, and after 40 days it was found in about half. By the long method, specific IgM was found in 27 of 32 sera taken between 2 and 117 days after onset. In the remaining 5 sera taken between 50 and 72 days after onset, there was HAI in the IgM fraction, but there was less than a 3-fold fall in titre after treatment with 2ME. By the short method, the IgM fractions of 5 sera (taken 9, 11, 21, 50 and 65 days after onset) showed only a 3-fold fall in titre after treatment with 2ME. All the other sera showed a 4-fold or greater fall.

The residual antibody found after 2ME treatment was considered to be IgG brought down by the IgM. The amount of this residual antibody was greater the higher the total HAI titre of the whole serum, and the higher the rubella-specific IgM titre. It was never found where there was no IgM, or in early sera where there was little or no IgG.

Specific IgM titres among the pregnant patients were similar to those of the non-pregnant patients.

Normal adults

Sera were collected from 16 laboratory staff, 7 of whom were known to have possessed rubella antibody at least a year previously, 5 at least 2–3 years previously, and 4 at least 7 years previously. None had detectable rubella-specific IgM by the short method. Nine were tested by the long method and none had specific IgM, or any HAI in the IgM fraction.

Rheumatoid factor

Sera from 6 patients known to contain rheumatoid factor all had fairly high levels of rubella HAI antibody. Rubella-specific IgM was detected in none by the short method.

DISCUSSION

Ultracentrifugation in a sucrose gradient followed by rubella HAI of the fractions was first described by Vesikari & Vaheri (1968) and later by Best *et al.* (1969). Both these groups of workers tested every fraction and Vesikari & Vaheri treated the sera with 2ME *before* fractionation to control the test. However, this is not a good control, since trailing will be eliminated in the control preparation but not in the test. Best *et al.* (1969) found specific IgM in all sera up to 20 days after onset, in a proportion of sera taken between 21 and 30 days and in none taken after 30 days. The test was simplified by Desmyter *et al.* (1971), who examined two fractions but detected no IgM after 36 days. Forghani *et al.* (1973) also tested only two fractions, and found specific IgM in all of 18 sera taken between 3 and 20 days after onset, and none of 10 subjects taken 1–30 years after rubella. A simplified test with only one fraction tested with and without 2ME similar to the one described here was developed by Field & Murphy (1972). In 6 patients with proved rubella, they found specific IgM in all of 8 sera taken 3–15 days after onset.

In the present study, the simplified method for detecting rubella-specific IgM worked well. It was not too time-consuming and the treatment of the fraction with 2ME gave little extra work. By the short method, IgM was found in all 49 sera taken between 5 and 30 days after onset, and in the 2 sera taken 40 days after onset. Thus there were no 'false negative' results, so that, when investigating patients with undiagnosed rashes on single late sera, failure to detect rubella-specific IgM between 5 and 30 days after onset means that it is unlikely that the illness is rubella.

There were also no 'false positive' results by the short method, in that 16 people known to have had rubella more than a year before showed no rubella-specific IgM. Six patients with rheumatoid factor, which is an IgM antiglobulin, and so can give false positive results by other techniques (Fraser, Shirodaria & Stanford, 1971; Cradock-Watson *et al.* 1972), showed no specific IgM by the present technique, although all had rubella antibodies. Desmyter (1972) likewise showed no false positive results with rheumatoid sera on sucrose gradients. It was found that in the concentration used in the present experiments, 2ME did not break down IgG and therefore give false positive results, for in all the IgG fractions in the calibration experiments, 2ME did not reduce the HAI titre, and in previous experiments with large numbers of whole sera from normal adults, treatment with 2ME did not affect the HAI titre. Cradock-Watson, Ridehalgh, Bourne & Vandervelde (1973) sometimes found IgA in the IgM fractions and suggested that early IgA might be partly in the polymeric form. This might give a false positive result, but would still indicate a recent infection. In the present work, IgA was not found in the IgM fraction.

It is possible that if a patient's serum continued to have rubella-specific IgM for years after a primary infection, on rare occasions this might give a false positive result. Specific IgM occasionally persists in patients with chronic rubella infections such as thrombocytopaenia or carpal tunnel syndrome for up to 9 months (Haire & Hadden, 1970), or after vaccination (Gupta, Peterson & Murphy, 1972). Baublis & Brown (1968) and Desmyter *et al.* (1971) have described the occasional persistence of IgM for more than 6 months in mothers carrying a rubella-infected fetus.

The long technique described here is probably less helpful than the short, since, although more of the sera taken more than 40 days after onset showed specific IgM than by the short method, and although there were no positive results several years after rubella, it is possible that the technique detects low levels of IgM many months after the onset of a straightforward infection, so that the finding of a low level by this technique is difficult to interpret. An investigation of sera taken between 2 and 24 months after infection is in progress.

The long technique might be of value in detecting IgM following administration of rubella vaccines inadvertently during pregnancy, particularly when vaccinees present many weeks after vaccination.

In this laboratory, the short technique has been used on pregnant patients who had had a rash or been in contact with rubella, and has successfully distinguished 9 who had recently had rubella from 61 who almost certainly had not.

Recently, reinfection with rubella after either wild rubella or after rubella vaccination has been reported. During reinfection the fetus is probably less likely to be affected than during primary infection. It is unlikely that rubella-specific IgM would be found during reinfection, so the test would be helpful in these cases.

The short method sometimes failed to detect rubella-specific IgM less than 5 days after onset, but this does not matter in practice for sera can always be taken a few days later to detect a rise in HAI or complement-fixing antibody.

Since IgA is also found early after an infection, some workers have suggested that the detection of rubella-specific IgA would also help in these patients (Bürgin-Wolff *et al.* 1971; Cradock-Watson *et al.* 1972). By the present technique, IgA could not be separated from IgG. Field & Murphy (1972) also could not separate them by sucrose density centrifugation. Although Bürgin-Wolff *et al.* (1971) and Cradock-Watson *et al.* (1972) found that rubella-specific IgA disappeared at about the same time as IgM, Ogra *et al.* (1971) using selective absorption with anti-IgG and IgM found that specific IgA was still present at fairly high titre a year after infection. Tests for rubella-specific IgA might therefore not be helpful in the field.

Sucrose gradient centrifugation for the detection of rubella-specific IgM has been criticized by Newman, Horta-Barbosa & Sever (1969) and by Sever (1969) because of trailing of IgG in the IgM fractions, which is particularly marked when undiluted serum is used (Best & Banatvala, 1969; Desmyter *et al.* 1971; Field & Murphy, 1972). In the present work, some trailing occurred especially where there was a high titre of both rubella-specific IgM and IgG. IgG may also be mixed with the lower fractions by bad technique. Both trailing and mixing are controlled by treating the fractions with 2ME, which shows that the inhibition is due to

IgM and not IgG. Forghani *et al.* (1973) suggested that 2ME is not satisfactory for confirming IgM in fractions because reaggregation may take place, but Field & Murphy and ourselves have found no difficulty in confirming IgM in the fractions with 2ME.

It is important to remove chick haemagglutinins before doing the test, but not non-specific inhibitors, since these remain at the top of the gradient and so do not interfere with the test. It is important not to use kaolin, since this may remove IgM. IgM is said to be inactivated at 56° C. and on storage at −20° C. In the present work, the titre of specific IgM was unaffected by 56° C. for 30 min. on one occasion, and sera which had been stored for up to 18 months at −20° C. often had high titres of specific IgM. The sucrose did not interfere with the HAI test, although the RBC took longer to settle, especially if they were old. This was overcome by reading all tests at 3 hr. instead of 2.

Rubella-specific IgM can be detected by immunofluorescence of cells infected with rubella examined with patient's serum and conjugated anti-human IgM serum (Baublis & Brown, 1968; Cohen *et al.* 1968; Haire & Hadden, 1970, 1972; Cradock-Watson *et al.* 1972, 1973). The best results were obtained by Haire & Hadden (1972), who found specific IgM in all sera up to 30 days after onset, in a proportion between 44 and 50 days and in none after 52 days. Sera containing rheumatoid factor give false positive results by this technique (Fraser *et al.* 1971) and another difficulty is the blocking of antigen by IgG antibody (Cradock-Watson *et al.* 1972). Specific fluorescence can be obtained only when the virus has been grown under special conditions (Haire, Adair & Fraser, 1972).

The separation of IgM on a Sephadex column and testing it for rubella HAI antibody with and without treatment of the fraction with 2ME was developed by Gupta *et al.* (1971, 1972) and used by Pead (1974). This technique requires less expensive equipment than sucrose density separation, but non-specific inhibitor may be a problem, and the fractions must be concentrated, and only one specimen can be processed each night.

Separation of IgM by filtration through agarose with rubella HAI of the fractions was described by Bürgin-Wolff *et al.* (1971), who detected specific IgM in all patients up to 18 days after onset and in a proportion of patients up to 11 weeks after onset.

Radioimmunodiffusion after sucrose gradient fractionation is probably the most sensitive method for the detection of rubella-specific IgM (Ogra *et al.* 1971). These workers found specific IgM in all patients with rubella up to 1 month from onset and in the majority up to 2 months. They found it in only 1 of the 25 patients at 4 months, and none at 6–8 months, so that the test is not too sensitive. Unfortunately, the technique requires equipment more specialized than is found in most routine diagnostic laboratories.

The only workers to compare two methods are Forghani *et al.* (1973). They found all of 18 sera taken between 3 and 20 days after onset were positive by both sucrose density separation with HAI and by immunofluorescence. However, there was no correlation between the titres obtained by the two tests. They considered that immunofluorescence was a more difficult test to do than sucrose density

separation. The simplified sucrose technique described here seems to be more sensitive than the techniques described by others except Ogra *et al.* (1971). It is comparatively simple and as many as 5 specimens can be tested per night.

We would like to thank the Public Health Laboratories at Bath, Exeter, Gloucester, Taunton and Salisbury, and many family doctors for the collection of the sera. The sera were collected during a rubella investigation by the PHLS Advisory Committee on Viral Reagents. We would also like to thank Mrs S. Hobbs for technical help, Mr N. Farran for the rheumatoid sera, and Dr H. R. Cayton and Dr S. J. Richmond for helpful advice.

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Some properties of calciferol as a rodenticide*

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SUMMARY

The potentiality of calciferol (alone and combined with warfarin) for the control of commensal rats and mice has been examined in the laboratory. Nearly all animals fed on 0·1 % calciferol for 2 days died. Though illness usually reduced food intake after the first 24 hr. there was no sign of aversion to the poison at 0·1 % – which is considered to be the lowest concentration suitable for use against *Rattus norvegicus*, *R. rattus* and *Mus musculus* in the field. There was some indication that resistance to warfarin in *R. norvegicus* may be correlated with susceptibility to calciferol. Toxicity tests with calciferol combined with warfarin indicated an additive effect between the compounds. No evidence for synergism was found however, although elsewhere there is some evidence for this.

INTRODUCTION

One of the problems that has confronted agriculturalists, public health authorities and others in Britain during the last decade has been the development of populations of rats and mice resistant to the widely used anticoagulant rodenticides such as warfarin (Greaves, 1971). In an attempt to solve the problem this laboratory has pursued collaborative research with the chemical industry with the object of identifying and developing new rodenticides. The present paper describes a laboratory investigation of calciferol (vitamin D_2). Study of this compound was prompted by data presented by Mr M. R. Hadler of Sorex (London) Ltd. regarding its promise against warfarin-resistant rats and mice and by the knowledge that his company would soon be marketing it in a formulation that also contained warfarin.

The action of calciferol is to raise blood calcium levels by stimulating the absorption of calcium from the intestine and mobilizing skeletal reserves. This takes many hours to build up to an effective level and the period of latency between the ingestion of calciferol and the development of hypercalcaemia has particular relevance to the effectiveness of the compound as a rodenticide.

METHODS

Wild rodents were used in nearly all the tests to be described and they included warfarin-resistant and non-resistant representatives of the three important commensal species, the Norway rat (*Rattus norvegicus*), the ship or roof rat (*Rattus rattus*) and the house mouse (*Mus musculus*). Males and females were employed in approximately equal numbers. Warfarin-resistant Norway rats were trapped on farms in Montgomeryshire, Shropshire and Kent and non-resistant rats caught by hand in a refuse destructor. Ship rats, both resistant and non-resistant, were trapped in dockland areas of Merseyside and London. Resistant house mice were caught by hand in agricultural premises in Hampshire and Nottingham, while non-resistant mice were bred in captivity from wild-caught parents. The 'non-resistant' animals were actually only presumed to be such on the basis that they were drawn from populations where to the best of our knowledge warfarin-resistance was not present. Resistant animals were classified as such after survival of a test exposure to warfarin, viz. a single subcutaneous dose of 200 mg./kg. of warfarin in dimethyl formamide (Norway rat), or unrestricted feeding on a sole diet of oatmeal containing 0·025 % warfarin for 21 days (house mouse) or 28 days (ship rat). As these methods of classification were empirical and to some extent arbitrary, it is possible that a few animals, particularly among the resistant Norway rats and the non-resistant ship rats may have been wrongly characterized.

Except where stated, animals were caged singly and maintained on Diet 41B for at least 2 weeks before being weighed within a few days of testing. Resistant animals were permitted to recover from their warfarin pre-treatment for a minimum of 2 weeks before being given calciferol. Feeding tests were of two kinds. First, there were tests in which the rodents were allowed to feed without restriction on a diet solely of poisoned bait for a specified number of days, after first being given the same bait unpoisoned in the experimental food pots for a few days. Second, there were tests in which animals were presented with a choice between plain and poisoned baits; fresh food pots were provided and the positions of the two baits interchanged each day. Any animal that rejected both of the baits on the first day was eliminated from the test. In all feeding tests bait consumption was measured regularly, usually each day, and fresh bait provided. Animals were maintained on Diet 41B for a 2-week observation period after withdrawal of the experimental baits and mortality recorded daily.

To study the possibility of synergism between warfarin and calciferol, a comparison was made of the subacute toxicity of the compounds administered separately and in combination. Male LAC Grey mice were grouped five to a cage and the compounds administered as four daily doses by stomach tube, using 5 % gum acacia as the vehicle. Dosage levels and the ratio of the two compounds in the mixture (warfarin: calciferol = 7:4) were chosen on the basis of preliminary assays, in such a manner that if the hypothesis that the toxicities were merely additive were correct, the 4-day log LD 50 of the mixture would lie between those of the constituent compounds (see Finney, 1971).

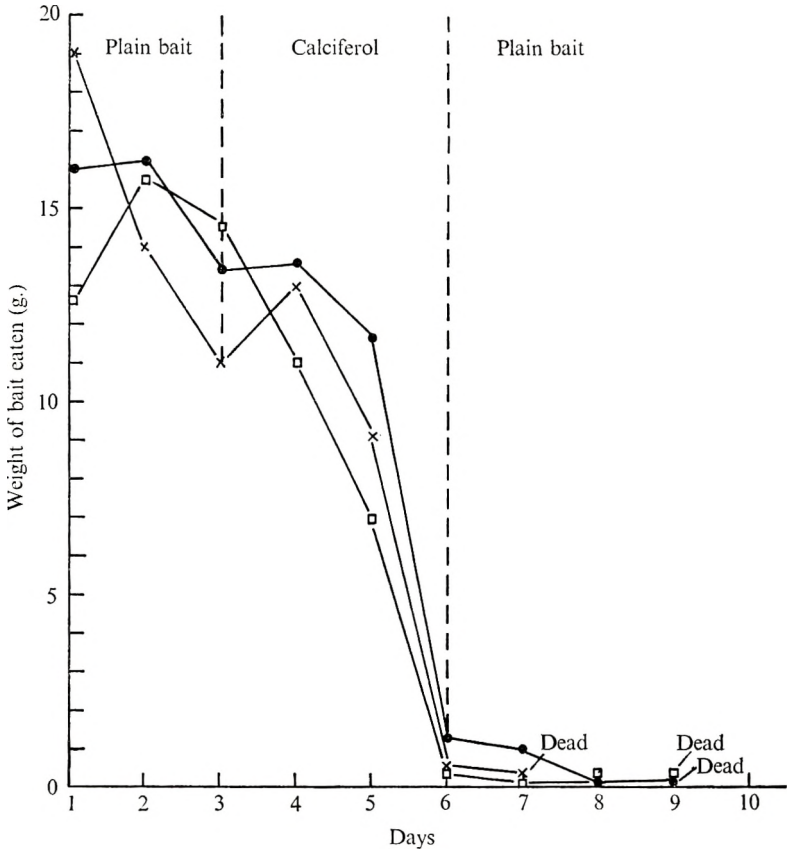

Fig. 1. Individual feeding figures for 3 wild non-resistant *R. norvegicus* given 0·1 % calciferol in pinhead oatmeal/corn oil bait for 3 days.

In the majority of feeding tests the poisoned bait was prepared by dispersing a wheat flour premix of calciferol (99 % purity, B.P. grade) and/or warfarin (99 % purity) at 5 % in medium oatmeal. In tests of a proposed field formulation a proprietary warfarin premix (fine oatmeal containing 0·5 % warfarin and 1·0 % Chlorazol Sky Blue FF200) was added to an equal amount of a corn oil solution of technical grade calciferol (67 % purity) and the resulting slurry thoroughly mixed at 10 % with medium oatmeal. Other baits in which corn oil was included were prepared in a similar manner. Plain bait was prepared in the same way as the poisoned bait for each test, the proprietary warfarin premix being replaced by wheat flour.

RESULTS AND DISCUSSION

Speed of rodenticidal action

In preliminary tests in which doses of 100 mg./kg. of calciferol were given on one or more days by stomach tube, laboratory rats and mice became visibly ill within 3 days. In feeding tests the onset of illness tended to curtail bait intake slightly on the 2nd day and severely on the 3rd day. Few animals ate more than a fraction of a gramme of bait on each subsequent day, and deaths usually ensued

Table 1. *Mortality and bait consumption by wild rodents given a sole diet of calciferol in medium oatmeal for 2 days*

Species	Type of animal	Mean body weight (g.)	Sex	Concentration of calciferol (%)	Mortality	Mean daily bait intake (g.)		Lethal dose of active ingredient (mg./kg.)		Survived dose of active ingredient (mg./kg.)		Days to death	
						Pre-bait*	Poison	Mean	Range	Mean	Range	Mean	Range
Rattus norvegicus	Non-resistant	250	M	0·1	5/5	16·2	11·8	95	82–104	—	—	4·2	4–5
		198	F		5/5	12·3	11·8	115	84–153	—	—	6·2	4–10
		244	M	0·05	4/5	17·4	16·0	65	53–75	67	—	5·5	5–7
		175	F		5/5	13·9	12·6	74	52–104	—	—	5·4	4–7
		260	M	0·025	1/5	15·7	14·2	37	—	26	20–33	8·0	—
		198	F		3/5	16·8	13·0	29	26–32	43	35–51	5·3	4–6
		271	M	0·0125	0/5	20·0	17·5	—	—	16	16–18	—	—
		178	F		0/5	13·5	13·6	—	—	20	11–24	—	—
		267	M	0·00625	0/5	14·6	13·1	—	—	6	5–8	—	—
		185	F		0/5	14·8	13·5	—	—	9	8–11	—	—
	Resistant	251	M	0·1	5/5	16·9	14·1	101	18–193	—	—	4·4	4–6
		247	F		5/5	16·1	13·0	106	83–133	—	—	4·6	4–6
		169	M	0·05	5/5	11·3	11·4	76	50–109	—	—	4·0	3–5
		142	F		3/5	13·5	10·8	74	61–90	85	84–85	5·0	4–6
		268	M	0·025	7/10	16·0	15·0	30	23–48	28	22–40	4·6	4–5
		217	F		5/10	15·8	13·9	33	27–41	32	25–42	5·6	4–10
		214	M	0·0125	1/5	15·6	16·8	18	—	21	17–25	6·0	—
		165	F		0/5	13·3	11·9	—	—	19	10–26	—	—
		196	M	0·00625	2/5	12·2	13·1	7	6–9	10	7–12	4·0	4
		223	F		3/5	17·0	14·7	7	6–9	12	10–13	4·7	3–7
Rattus rattus	Non-resistant	153	M	0·1	5/5	12·2	8·2	112	89–141	—	—	6·6	5–9
		107	F		5/5	11·2	8·0	156	89–201	—	—	5·8	5–7
		194	M	0·05	5/5	16·7	15·1	92	48–185	—	—	4·4	3–5
		178	F		4/5	20·6	13·6	69	59–83	100	—	3·3	2–4
		194	M	0·025	4/5	13·4	13·0	33	21–40	34	—	4·0	3–7
		115	F		3/5	13·0	11·8	49	36–68	67	62–73	4·3	3–6
		177	M	0·0125	5/5	15·8	14·2	21	15–26	—	—	6·4	4–9
		188	F		2/5	18·4	13·1	16	15–16	19	16–23	5·0	4–6
		203	M	0·00625	0/4	13·8	13·3	—	—	9	6–9	—	—
		144	F		0/5	12·3	11·5	—	—	10	9–11	—	—

* Last day only.

Table 1 (*cont.*)

Species	Type of animal	Mean body weight (g.)	Sex	Concentration of calciferol (%)	Mortality	Mean daily bait intake (g.)		Lethal dose of active ingredient (mg./kg.)		Survived dose of active ingredient (mg./kg.)		Days to death	
						Pre-bait*	Poison	Mean	Range	Mean	Range	Mean	Range
	Resistant	175	M	0·1	3/5	12·0	10·1	120	101–145	118	103–133	5·0	5
		161	F		4/5	6·2	5·4	83	40–149	12	—	5·5	4–7
		197	M	0·05	1/5	10·0	15·1	60	—	32	20–44	4·0	—
		160	F		2/5	10·0	11·0	58	58–59	25	9–42	6·5	3–10
		199	M	0·025	2/5	8·9	9·9	22	18–24	29	28–30	4·0	4
		156	F		2/5	9·3	9·4	33	29–38	30	25–35	7·0	5–9
		193	M	0·0125	0/5	7·3	9·7	—	—	13	7–21	—	—
		153	F		1/5	9·4	12·6	21	—	19	10–26	4·0	—
		161	M	0·00625	0/5	11·2	8·9	—	—	9	3–18	—	—
		175	F		0/5	7·3	5·7	—	—	4	2–6	—	—
Mus musculus	Non-resistant	21	M	0·1	5/5	2·5	2·6	252	212–277	—	—	6·2	4–11
		14	F		5/5	2·1	2·2	312	279–369	—	—	6·0	5–8
		19	M	0·05	5/5	2·2	2·7	138	128–159	—	—	6·0	6
		13	F		4/5	2·1	2·2	162	139–173	186	—	6·0	6
		16	M	0·025	4/5	1·7	2·1	67	53–84	65	—	6·5	6–7
		11	F		4/5	1·9	1·8	94	83–103	50	—	6·0	6
		19	M	0·0125	0/5	3·0	3·4	—	—	44	42–47	—	—
		12	F		1/5	2·4	2·2	39	—	50	44–61	6·0	—
		16	M	0·00625	0/5	2·7	3·0	—	—	25	20–28	—	—
		11	F		0/5	2·4	2·4	—	—	28	23–39	—	—
	Resistant	18	M	0·1	5/5	3·9	2·9	330	265–400	—	—	4·2	4–5
		14	F		5/5	3·6	3·6	500	346–587	—	—	4·4	3–7
		17	M	0·05	2/5	3·7	3·2	188	181–195	184	122–234	7·0	5–9
		11	F		5/5	2·6	2·5	240	217–265	—	—	4·6	4–5
		16	M	0·025	0/5	3·5	2·9	—	—	91	91–103	—	—
		13	F		1/4	3·5	3·2	110	—	129	129–145	6·0	—
		16	M	0·0125	0/5	4·0	3·2	—	—	50	44–58	—	—
		11	F		0/5	2·7	2·5	—	—	56	49–66	—	—

* Last day only.

by the 7th day. The typical pattern is illustrated by the data for three rats in Fig. 1.

Illness induced by a toxic diet sometimes predisposes animals to avoid that diet subsequently – a phenomenon usually termed 'bait shyness' or 'poison shyness'. A theoretical consequence in field treatments with calciferol could be that, if some rodents failed to ingest a lethal dose within the first 2 days, they might then tend to turn to an alternative, non-toxic diet. In the laboratory such considerations make the interpretation of results of prolonged feeding tests problematical, and it was therefore decided to limit the majority of tests to a duration of 2 days.

Toxicity of bait containing calciferol only

The results of feeding tests in which animals were offered calciferol bait for 2 days are summarized in Table 1. As a result of the gradual onset of illness towards the end of the 2nd day the mean daily consumption of calciferol bait, particularly at the highest concentration of 0·1 % was usually rather less than the last day's consumption of unpoisoned pre-bait. However, it can be seen from the last two columns but one of Table 1 that survivors often ate more calciferol than did animals that died, indicating that survival was not associated with any aversion to the compound.

At a concentration of 0·1 % calciferol surpassed all lower concentrations, producing complete mortality in nearly every group that received it. The concentration of 0·1 % is therefore proposed as the lowest that should be considered for use in rodenticidal bait in the field. Warfarin-resistant ship rats were the only animals to survive feeding for 2 days on 0·1 % calciferol and it may well be expedient to increase the concentration further for use against animals in this category. The fact that mortality in warfarin-resistant ship rats and house mice was generally less than in non-resistant animals of the same species suggests that warfarin resistance in these species may be due to a relatively generalized detoxification mechanism rather than to a change in metabolism that affects warfarin specifically. There is also a suggestion in the greater mortality that occurred among warfarin-resistant Norway rats as compared with their non-resistant counterparts, that the former are the more sensitive to calciferol poisoning, possibly owing to the tendency of these animals to suffer from spontaneous vitamin K deficiency (Greaves & Ayres, 1973). The possibility that resistance to warfarin may be positively correlated with susceptibility to calciferol in the Norway rat is of obvious practical importance and deserves to be investigated further.

Toxicity of mixtures of calciferol and warfarin

The results of various tests conducted with bait containing both warfarin at 0·025 % and calciferol at 0·1, 0·025 and 0·01 % are summarized in Table 2. With calciferol at 0·1 % for 2 days (Groups 1–4) there was complete mortality in all three species except for the survival of a single warfarin-resistant ship rat. With calciferol at 0·025 % for 4 days, 10/10 warfarin-resistant Norway rats also died. But when the concentration of calciferol was as low as 0·01 % complete mortality was not attained with warfarin-resistant Norway rats (Group 6) even after a

Table 2. *Mortality and bait consumption by wild rodents given a sole diet of bait containing calciferol and/or warfarin for a limited number of days*

Type of animal	Group	Mean body weight (g.)	Feeding periods (days)	Concentration of poison (%)		Mortality	Mean daily bait intake (g.)		Days to death	
				Calciferol	Warfarin		Pre-bait†	Poison	Mean	Range
R. norvegicus (resistant)	1*	201	2	0·1	0·025	10/10	—	6·2	4·7	3–6
M. musculus (non-resistant)	2*	15	2	0·1	0·025	10/10	—	1·7	3·2	3–4
M. musculus (resistant)	3*	14	2	0·1	0·025	10/10	—	1·5	3·9	3–6
R. rattus (resistant)	4*	140	2	0·1	0·025	9/10	13·6	7·1	5·8	4–11
R. norvegicus (resistant)	5	135	4	0·025	0·025	10/10	14·8	6·1	4·3	3–7
R. norvegicus (resistant)	6	226	28	0·01	0·025	12/14	18·8	9·2	8·5	4–20
R. norvegicus (non-resistant)	7	185	2	0·0125	—	0/10	10·7	12·6	—	—
		229	2	—	0·003125	2/10	11·9	13·6	7·0	6–8
		203	2	0·0125	0·003125	7/9	9·0	11·6	7·6	6–12
M. musculus (non-resistant)	8	12	2	0·0125	—	1/10	2·4	2·2	5·0	—
		12	2	—	0·003125	3/10	2·9	2·1	4·7	4–6
		11	2	0·0125	0·003125	0/10	2·6	2·1	—	—

* Bait contained 5% corn oil. † Last day only.

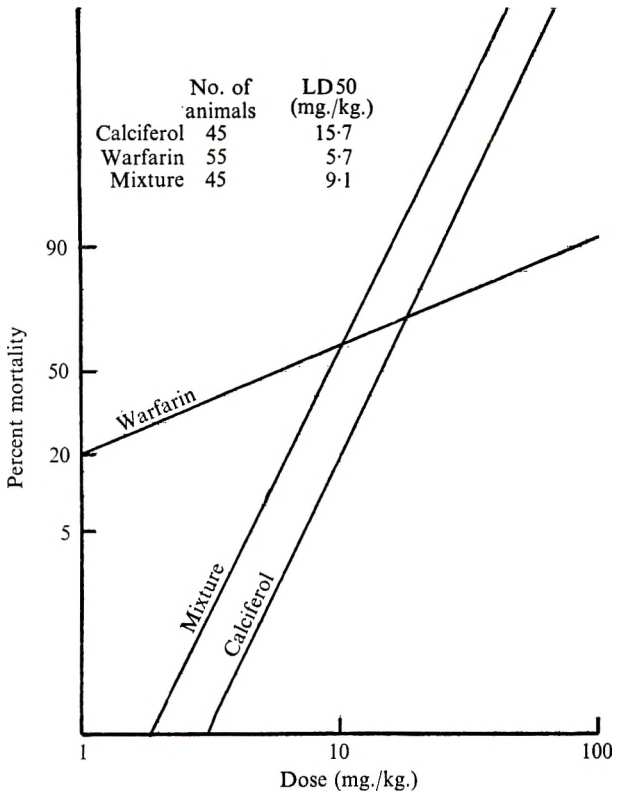

Fig. 2. Subacute oral toxicity of warfarin, calciferol and a 7:4 mixture to LAC Grey mice.

feeding period of 28 days. However, the latter animals (Group 6) were survivors of a pilot field treatment in which exactly the same bait formulation was used as in the laboratory test, and their susceptibility may therefore have been atypically low; their feeding behaviour while on test was of some interest in that the daily bait intake of several rats decreased and increased intermittently as they became ill and recovered in varying degrees. Taken together, the above results indicate that warfarin/calciferol mixtures are no less toxic than calciferol alone.

A not uncommon practical difficulty in field treatments is that rodents may continue to utilize their normal food sources, feeding only partly from the rodenticidal bait that is offered. In an attempt to mimic a low level of bait consumption in the laboratory, bait containing 0·0125 % calciferol and 0·003125 % warfarin (i.e. one-eighth of the candidate field concentration of 0·1 % calciferol and 0·025 % warfarin) was given to non-resistant Norway rats and house mice for 2 days. Two further groups of each species were given bait containing calciferol or warfarin separately, for comparative purposes. The results are given in Groups 7 and 8 of Table 2. The tests with 0·0125 % calciferol alone duplicated the low kills given for this concentration in Table 1, and warfarin also gave low mortalities. In comparison the mixture produced a marked increase in mortality in Norway rats suggesting at least an additive effect between the two compounds.

Table 3. *Bait consumption and mortality in wild* R. norvegicus *and* M. musculus *given a choice between plain and poisoned baits containing* 5 % *corn oil*

Species	Group	Mean body weight (g.)	Duration of test (days)	Concentration of poison (%)		Mean daily bait intake (g.)*		Significance (P) of Student's 't'	Mortality
				Calciferol	Warfarin	Plain	Poisoned		
R. norvegicus	1	253	4	0·025	—	9·0	11·3	< 0·7	3/5
	2	266	4	0·25	—	14·2	6·1	< 0·02	5/5
	3	234	4	0·1	0·025	10·9	6·4	< 0·1	10/10
	4	221	4	0·025	0·025	7·9	5·2	< 0·05	8/10
M. musculus	5	16	4	0·025	—	1·6	1·6	1·0	5/5
	6	14	4	0·25	—	1·2	0·7	< 0·01	5/5
	7	16	4	0·1†	0·025	1·2	0·8	< 0·3	9/9
	8	12	5	0·1†	0·025	1·2	0·8	< 0·2	9/9

* During the first two days of each test.

† Active ingredients in the form of proprietary concentrates.

With house mice however the kill with the mixture was below that obtained with either compound given separately. To follow up this curious observation in conditions more reproducible than could be provided in a feeding test, the compounds were administered separately and together to LAC Grey mice by stomach tube. The results are summarized in Fig. 2. The difference between the slopes of the lines for warfarin and calciferol precludes an analysis of relative potency, though it may be noted that, in terms of slope, the mixture resembles calciferol rather than warfarin. At the LD50 level the mixture is intermediate in toxicity between calciferol and warfarin, indicating an additive effect at this point.

We do not consider that the present results provide a basis for suggesting that any synergistic or other interaction between warfarin and calciferol exists. However, this subject also might repay further investigation, for Mr M. R. Hadler (private communication) reports that in oral intubation tests with *R. norvegicus* heterozygous for warfarin resistance, calciferol at 10 mg./kg. plus warfarin at 20 mg./kg. in polyethylene glycol administered on each of 3 days killed 8/8 animals – whereas the results of administering calciferol at 10 mg./kg. alone and warfarin even at 50 mg./kg. were 0/5 and 1/8 dead, respectively.

In similar tests with *Mus musculus* calciferol plus warfarin, both at 10 mg./kg., killed 10/10 animals although the two poisons separately killed 4/10 and 0/10 respectively. A complete kill (10/10) was also obtained with the two poisons each at 5 mg./kg. – compared with kills of 2/10 and 1/10 respectively when calciferol and warfarin were used alone at the same concentration.

It may be noted in passing that partial reversal of prothrombin deficiency by vitamin D has been reported (Elliott, Isaacs & Ivy, 1940); contamination with vitamin K is a possibility which must be considered in relation to this report, but taken at its face value it suggests an antagonistic action between warfarin and calciferol. Against this, it seems possible that the vascular lesions familiar in chronic hypervitaminosis D (Hass, Trueheart & Hemmens, 1960) may increase the likelihood of haemorrhage in the presence of warfarin. In the brief post-mortem examinations performed in the present study, though haemorrhages were occasionally seen in animals that had received warfarin, the pale liver and massive bleeding typically associated with death from anticoagulant poisoning was something of a rarity, and it was concluded that hypercalcaemia, occasionally made apparent by gross calcification of the aorta, was more often the cause of death.

The palatability of calciferol baits

The results of tests in which Norway rats and house mice were given a choice for 4 or more days between plain and poisoned bait are summarized in Table 3. Only the first 2 days' bait consumption were taken as an unbiased measure of palatability, since thereafter the toxic effects of calciferol reduced feeding differentially, depending upon the degree of preference each individual animal had shown for the poisoned bait earlier in the test. Bait containing 0·025 % calciferol was well accepted by both rats and mice (Groups 1 and 5), and even when the concentration was increased tenfold (Groups 2 and 6) still accounted for about a third of the bait consumed, confirming that calciferol is not particularly unpalatable at rodenticidal

concentrations. Bait containing 0·1 % calciferol and 0·025 % warfarin was also reasonably well accepted by both species, accounting for rather more than a third of the bait consumed (Groups 3, 7 and 8).

CONCLUSIONS

The above results indicate that the minimum concentration at which calciferol, either alone or combined with warfarin, is likely to give good results in the field against rats or mice is 0·1 %. One pre-eminent advantage that calciferol has is that it is toxic to warfarin-resistant rodents. A second advantage is that it kills more rapidly, within 1 week instead of the 1–3 weeks that are often required with anticoagulants, particularly against the ship rat and house mouse. This more rapid action means however that the time available for the target species to ingest a lethal dose (i.e. before the onset of illness curtails feeding) is limited to little more than 2 days. Misjudgements in making the initial bait placements in field treatments with calciferol would therefore be more likely to lead to sublethal dosing of the rodents. Considerable field experience may be necessary to ascertain whether this would be a significant disadvantage in practice and, indeed, whether calciferol should best be employed as a rodenticide in its own right or in conjunction with anticoagulants. One argument that has been advanced for the latter practice is that where two poisons are used the likelihood is less that resistance to either one of them would develop. This assumes that resistance to calciferol would include the non-development of the fall-off in feeding that the compound normally induces after about 24–48 hr.

Warfarin is generally regarded as a particularly safe rodenticide, largely because it is a cumulative poison and the successive doses required to build up lethal levels are not easily acquired by accident. To the extent that calciferol is cumulative in action it may be considered to have the same advantage from the standpoint of safety in use. However, regular users of rodenticidal formulations of calciferol should be alert to the danger of chronic hypervitaminosis D that could result from careless handling.

As a final practical point it may be noted that calciferol tends to decompose in the presence of air and moisture, and the toxicity of damp calciferol baits has been found to decrease significantly within a week. The long-term stability of corn oil solutions is satisfactory however and, contrary to what had been supposed previously, dry cereal/corn oil bait retains its rodenticidal activity for some months.

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Field trials of calciferol combined with warfarin against wild house-mice (*Mus musculus* L.)*

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SUMMARY

A combination of calciferol (vitamin D_2) and warfarin, each at 0·025 % in medium oatmeal bait, failed to control six of seven house-mouse (*Mus musculus* L.) populations infesting urban and farm buildings. In three further treatments with both calciferol and warfarin at 0·05 % in dehusked canary seed bait plus 5 % corn oil, mortality, estimated from the consumption of pre- and post-treatment census bait, ranged between 94·2 and 97·4 %. Finally, among sixteen treatments done with calciferol at 0·1 % and warfarin at 0·025 % in various cereal baits, the best results (97·0–100 %) were obtained in six treatments where the bait-base was whole canary seed; this was so whether the poison bait was applied directly or after a 3-day pre-baiting period. It is concluded that calciferol at 0·1 % plus warfarin at 0·025 % is an effective combination against house-mice, especially when used with whole canary seed. The role played by warfarin in the poison mixture needs to be investigated further.

INTRODUCTION

The difficulty that has been experienced in controlling rodent infestations in Britain during the past decade owing to the development of resistance to the anticoagulants has been referred to by Greaves, Redfern & King (1974). In an earlier study on the response of wild mice (*Mus musculus* L.) to various anticoagulants (Rowe & Redfern, 1968) attempts were made to render suspected warfarin-resistant animals more susceptible to poisoning by including either sulphaquinoxaline, SKF525-A or vitamin A acetate in 0·025 % warfarin bait. Only vitamin A acetate, in excess, was found to bring about a high mortality rate in the test animals but since its presence greatly lowered the acceptance of the warfarin bait the efficacy of the combined bait was not determined in the field.

More recent laboratory tests (Greaves *et al.* 1974) have shown that bait containing calciferol (vitamin D_2) alone and in combination with warfarin is both toxic and acceptable to wild mice, including warfarin-resistant animals. The present paper describes the results of poison treatments using calciferol and warfarin in combination, against free-living *M. musculus*. The treatments were carried out simultaneously with work on finding improved bait-bases for use against mice and this influenced the course of the tests to be described.

METHODS

The treatments were carried out in urban and rural areas but the majority were done against mice infesting farm storage buildings and animal-rearing houses. Although some of the chosen premises had histories of persistent mouse infestation, despite prolonged or frequent treatment with warfarin, no laboratory tests were carried out before the poison treatments were begun, to confirm that the mice inhabiting them were resistant to warfarin.

Three series of poison treatments were carried out. In the first (Series A), calciferol and warfarin were both included in bait at 0·025 %, the concentrations suggested for field evaluation by the manufacturers. The poison bait was prepared by thoroughly mixing nineteen parts of medium grade oatmeal with one part of a proprietary master-mix comprising 0·5 % calciferol and 0·5 % warfarin in fine oatmeal.

In the second set of treatments (Series B) the concentration of calciferol used was 0·05 % and since at this time the compound was still only available in the form of the above master-mix, the concentration of warfarin had also to be increased to 0·05 %. This is double the strength normally recommended against mice. In each of the treatments the poison bait used was prepared by mixing one part of the proprietary master-mix to nine parts of dehusked canary seed treated with 5 % corn oil.

In the third set of treatments (Series C) one part of 2 % calciferol dissolved in corn oil was mixed with eighteen parts of bait and then one part of a 0·5 % warfarin master-mix was added while stirring continuously, to give a final formulation containing 0·1 % calciferol and 0·025 % warfarin.

With one exception the effectiveness of each Series A poison treatment was assessed by comparing the amounts of poison bait eaten, the number of visits made by mice to the baiting points and the level of activity (footprints and tail-swipes) revealed by strategically placed and regularly smoothed patches of an inert dust.

Percentage success in one of the Series A treatments and in the Series B and Series C treatments was estimated from the total amounts of plain bait that were eaten by mice at pre- and post-treatment censuses. Each treatment was conducted in the following manner. On day 1 the infested area was surveyed thoroughly and the pre-treatment census begun. For this purpose a plain cereal bait, the total weight of which was known, was laid in small, wooden trays distributed a few feet apart throughout the infested area. At sites frequented by birds the trays were protected by metal covers. The total amount of census bait eaten was measured daily for 4 days or, occasionally, at the end of the 4-day census period. In either case, each baiting point was inspected daily to ensure that surplus bait was always available.

At the end of the pre-treatment census the trays and surplus bait were removed and 3 days later the poison treatment was begun. The bait-base used for poisoning and the poison baiting sites were different from those employed in the census baiting; the poison bait was maintained in excess at each site throughout the

Table 1. *Consumption of poison bait in treatments against mice, using* 0·025 % *calciferol and* 0·025 % *warfarin in various bait-bases (Series A)*

Treatment no.	Days	Bait-bases	Consumption of poison bait (g.)
1	1–7	Medium oatmeal	111
	8–15	Medium oatmeal	19
2	1–7	Medium oatmeal	144
	8–15	Medium oatmeal	76
3	1–7	Medium oatmeal	111
	8–14	Medium oatmeal	37
	15–25	Medium oatmeal	28
	26–35	Pinhead oatmeal/corn oil/sugar	31
4	1–11	Medium oatmeal	39
	12–19	Dehusked canary seed/corn oil	18
5*a*	1–7	Medium oatmeal	195
	8–14	Medium oatmeal	106
Interval	15–18	Census baiting (plain)	
	19–21	No bait	
	22–25	Choice of plain baits	
5*b*	26–32	Wheat/porridge oats/dehusked canary seed/corn oil/sugar	756
	33–39	Wheat/porridge oats/dehusked canary seed/corn oil/sugar	336
	40–50	Wheat/porridge oats/dehusked canary seed/corn oil/sugar	409
6	1–7	Medium oatmeal	409
	8–14	Medium oatmeal	186
	15–46	Medium oatmeal	368
7	1–7	Medium oatmeal	638
	8–14	Medium oatmeal	96
	15–46	Medium oatmeal	210

treatment period and the total amount eaten was measured daily during the early stages of the treatment and then at less frequent intervals. At each visit the number of baiting points visited by mice was recorded and the dust patches were inspected for mouse signs. The treatment was terminated either when the poison bait was no longer being eaten or visited and the dust patches were undisturbed or when, after the first 7 days of the treatment, there was a regular, albeit small, take of poison bait over several days but little or no decline in activity in the dust patches.

At the end of the treatment period the poison bait and trays were removed and 3 days later, a post-treatment census, conducted in the same manner as the pre-treatment census, was begun.

RESULTS AND DISCUSSION

Series A treatments

Table 1 gives the amount of poison bait consumed during the seven treatments. In each case the greatest amount of medium oatmeal poison bait was eaten on

either day 2 or day 3 and, as Table 1 shows, there was a marked decline in consumption after day 7. In Treatments 1 and 2 however mice were active in the dust patches throughout the 15 days that poison bait was laid. This was also the case in Treatment 3, which was continued for 25 days. Here the dust patches were also crossed daily during a second 10-day poison treatment when pinhead oatmeal plus 5 % corn oil and 5 % sugar was used as bait. Inadequate control also resulted in Treatment 4 in which poisoned medium oatmeal was laid for 11 days. No poison bait was eaten after day 7 and a second treatment was carried out using bait comprising dehusked canary seed plus 5 % corn oil. More poison bait was consumed as a result but some mice still survived.

Treatment 5 was conducted in two parts. The take of treated medium oatmeal bait (301 g.) in an initial 14-day treatment period (5*a*) seemed low judging from the abundant signs of mice that were evident at the survey and this was confirmed by the amount of whole canary seed eaten (462 g.) in a 4-day post-treatment census baiting. After an interval of 3 days the mice were given a choice of three different plain baits over a 4-day period. A second 25-day poison treatment (5*b*) was then done using the most preferred bait (a mixture of equal amounts of whole wheat, porridge oats and dehusked canary seed plus corn oil and sugar, each at 5 %) as the carrier for the two poisons. The take of this bait during the first 14 days (1092 g.) was considerably greater than in the same period of the first treatment when medium oatmeal was used. However, although a further 409 g. of the mixed bait were eaten during the remaining 11 days of the treatment and 20 dead mice were found, the dust patches indicated that a considerable number of animals had survived. In a post-treatment census baiting 216 g. of whole canary seed were eaten in 4 days and the estimated kill in the second treatment therefore was only 53·2 %.

In Treatments 6 and 7 medium oatmeal poison bait was laid unchanged for 46 days. After day 2 in Treatment 6 the amount of poison bait eaten, the numbers of baiting points visited and activity in the dust patches all decreased as the treatment progressed. In census baitings carried out before and after the treatment, 252 g. and 6 g. of bait were eaten respectively (estimated treatment success 97·6 %). In Treatment 7 most poison bait was also eaten during the first 2 days and thereafter feeding occurred at a much lower level. Twenty-nine dead mice were found, the first on day 2, but despite the low consumption of poison bait at the end of the treatment period, the heavy traffic in the dust patches indicated a residual population of some size. The amount of canary seed eaten (291 g. in 4 days) in a post-treatment census baiting confirmed this conclusion.

Series B treatments

The seven treatments in Series A indicated both that the concentrations of calciferol and/or warfarin employed (0·025 %) were too low and that medium oatmeal was insufficiently attractive. Treatments 4 and 5*a* particularly emphasized the latter point. Effective control was obtained in Treatment 6 where limited alternative food was available, but even here the treatment lasted 46 days. In the three treatments comprising Series B therefore, the calciferol concentration

Table 2. *Consumption of poison bait in treatments against mice using* 0·05 % *calciferol and* 0·05 % *warfarin in dehusked canary seed/corn oil bait (Series B)*

Treatment no.	Pre-treatment census: consumption of pinhead oatmeal (g.)	Consumption of poison bait		Post-treatment census: consumption of pinhead oatmeal (g.)	Estimated success (%)
		Days	Quantity (g.)		
8	929	1–7	397	24	97·4
		8–14	77		
		15–17	8		
9	104	1–7	42	6	94·2
		8–14	10		
		15–18	1		
10	1246	1–7	367	48	96·1
		8–14	78		
		15–21	98		
		22–25	48		

was increased to 0·05 % and, for reasons explained above, so was the concentration of the warfarin. At the same time the bait-base was changed to dehusked canary seed, which laboratory tests had shown to be preferred to medium oatmeal.

The results of the three treatments in Series B are given in Table 2. Most poison bait was eaten during the first 2 days and, as also in the Series A treatments, consumption fell markedly after 7 days. Contrasting with all but Treatment 6 in Series A, however, the fall in consumption and number of visits to the baiting points that occurred as the treatments progressed was paralleled by a decline in activity in the dust patches. Even so, Column 6 of Table 2 shows that individuals survived each of the treatments, which were fairly prolonged (17–25 days).

In the next series of trials, therefore, it was decided to increase the concentration of calciferol further, to 0·1 %. The advisability of this was confirmed by results obtained later by Greaves *et al.* (1974) in the laboratory, and because a master-mix of calciferol separately in corn oil was now available, it was possible to include warfarin at 0·025 %.

Series C treatments

The first three treatments of Series C (11, 12 and 13 in Table 3) were again done with dehusked canary seed as the bait-base and estimated control figures of 91·0, 91·8 and 91·5 % were obtained. In the next five (14–18), the bait-base used was pinhead oatmeal, which also had shown up reasonably well in laboratory palatability tests. As in previous treatments, most poison bait was eaten during the first 7 days, but, as Table 3 shows, success varied from 29·6 to 91·6 %. Two further tests with pinhead oatmeal as the bait-base gave poor results over 17 days of feeding (Treatments 19*a* and 20*a*): but when, in 17-day follow-up treatments at the same sites, dehusked canary seed in one case (19*b*) and dehusked canary seed and mixed cereal in the other (20*b*), were substituted for the oatmeal, good control was then obtained (97·0 and 97·8 %, respectively). Unfortunately wet weather prevented meaningful monitoring of the course of feeding.

The final six treatments in Series C (Table 4) were done with whole canary seed

Table 3. *Consumption of poison bait in treatments against mice using* 0·1 % *calciferol and* 0·025 % *warfarin in various bait-bases (Series C)*

Treatment no.	Census bait	Consumption of pre-treatment census bait (g.)	Bait-base	Consumption of poison bait		Consumption of post-treatment census bait (g.)	Estimated success (%)
				Days	Quantity (g.)		
11	Pinhead oatmeal	2122	Dehusked canary seed	1–7	1072	192	91·0
				8–18	234		
12	Pinhead oatmeal	1366	Dehusked canary seed	1–7	595	112	91·8
				8–18	54		
13	Pinhead oatmeal	189	Dehusked canary seed	1–7	86	16	91·5
				8–21	79		
				22–27	25		
14	Whole canary seed	52	Pinhead oatmeal	1–7	28	10	80·8
				8–12	16		
15	Whole canary seed	155	Pinhead oatmeal	1–11	*	26	91·6
16	Whole canary seed	89	Pinhead oatmeal	1–7	17	27	69·7
				8–12	2		
17	Whole canary seed	26	Pinhead oatmeal	1	12	9	64·4
				2–12	*		
18	Whole canary seed	162	Pinhead oatmeal	1–7	50	115	29·6
				8–17	19		
19*a*	Whole canary seed	392	Pinhead oatmeal	1–7	76	280	28·6
				8–17	*		
19*b*	Whole canary seed	280	Dehusked canary seed	1–17	*	4†	97·0
20*a*	Whole canary seed	617	Pinhead oatmeal	1–7	87	389	37·0
				8–17	*		
20*b*	Whole canary seed	389	Wheat/porridge oats/dehusked canary seed	1–17	*	3†	97·8

* Unrecordable. † Porridge oats.

Table 4. *Consumption of poison bait in treatments against mice using* 0·1 % *calciferol and* 0·025 % *warfarin in whole canary seed bait (Series C)*

Treatment no.	Census bait	Consumption of pre-treatment census bait (g.)	Consumption of poison bait (g.)		Consumption of post-treatment census bait (g.)	Estimated success (%)
			Days	Quantity (g.)		
21	Pinhead oatmeal	670	1–7	1135	25	97·0*
			8–14	607		
			15–21	765		
			22–28	56		
			29	19		
22	Pinhead oatmeal	331	1–7	361	4	98·8*
			8–14	20		
			15	0		
23	Pinhead oatmeal	115	1–7	122	0	100*
			8–14	0		
			15	0		
24	Pinhead oatmeal	707	1–7	270	8	98·9
			8–11	5		
25	Pinhead oatmeal	376	1–7	213	0	100
			8–9	0		
26	Pinhead oatmeal	423	1–7	485	0	100
			8–14	0		
			15	0		

* Poisoning preceded by pre-baiting.

as the bait-base. This bait had shown up as well as the dehusked seed in palatability tests but it has the disadvantage that mice tend to dehusk it, and probably discard at the same time part or all of any poison adhering to it. Preliminary tests showed however that whole canary seed mixed with the 2 % solution of calciferol in corn oil and treated with warfarin master-mix was toxic to mice, presumably because enough calciferol found its way below the husks to significantly increase the toxicity of the formulation to mice. Poison bait prepared in this manner was used in Treatments 21–26.

In Treatments 24, 25 and 26 the poison bait was laid directly. In the other three treatments poison baiting was preceded by 3 days of 'pre-baiting' with a mixture of 90 % whole canary seed, 5 % corn oil and 5 % wholemeal flour.

Table 4 shows that five of the six treatments gave excellent results in from 9 to 15 days. In Treatment 21 good bait-takes in the first week did not result in much reduction in activity and adequate control was obtained only after 29 days. A contributory factor may have been that certain bait points were so well protected from farm stock that they were also largely ignored by the mice: for when on day 8 they were re-located and increased in number, feeding increased.

CONCLUSIONS

House-mice are most difficult to control in places where food and cover coincide and are extensive. The success of a poison treatment is then as much dependent on having an attractive bait-base as it is on using an effective poison. The results of the poison treatments reported here showed that, provided baiting problems can be overcome, calciferol at 0·1 % plus warfarin at 0·025 % can give effective control of *M. musculus*. The excellent results of the treatments using whole canary seed mixed with calciferol in corn oil and with warfarin also indicate that this formulation is most likely to succeed in controlling mice living in 'difficult' environments.

The present work however does not show either the extent to which warfarin contributes to the effectiveness of bait containing calciferol or how far calciferol penetrates below the husks when it is added in corn oil to whole canary seed. Both subjects are being investigated further.

Calciferol and warfarin were kindly supplied by Mr M. R. Hadler of Sorex (London) Ltd., who also made available the results of his own laboratory tests on mice. Our thanks are due to C. Plant, A. Bradfield and to other colleagues who assisted in this work and also to several local authorities for their co-operation.

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Field trials of calciferol against warfarin resistant infestations of the Norway rat (*Rattus norvegicus* Berk.)*

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SUMMARY

The effectiveness of calciferol (vitamin D_2) against *Rattus norvegicus* was investigated in field trials on twenty-three farms with rat infestations partly resistant to warfarin. At 0·01 % combined with warfarin at 0·025 % no rodenticidal effect of the calciferol was discernible. At 0·025 % with warfarin at the same concentration, results were better, but not appreciably better than is often obtained with warfarin alone against resistant rat populations. When the concentration of calciferol was stepped up to 0·1 %, four out of five treatments in which the poison was applied directly gave complete control. The fifth may have partly failed because of poison shyness caused by under-baiting. Five out of six more treatments done after 'pre-baiting' were also successful. The sixth failed for reasons unconnected with the choice of poison.

Six further infestations that were not responding adequately to warfarin treatments were quickly controlled when, in three instances, calciferol at 0·1 % was used instead and, in three more, it was used together with warfarin. It is concluded that calciferol at 0·1 % is an effective poison against *R. norvegicus* either combined with warfarin or not, but that because at 0·1 % its effect is subacute rather than chronic, there may be a case in some environments for using it only after pre-baiting.

INTRODUCTION

The rodenticidal potentialities of calciferol (vitamin D_2) alone and in combination with the anticoagulant warfarin have been discussed from the laboratory aspect by Greaves, Redfern & King (1974). The present paper describes the result of a number of field treatments in Montgomeryshire against *Rattus norvegicus* with calciferol, in most cases combined with warfarin, the form in which the manufacturer developing calciferol was proposing to market it when the tests began. And since, as Greaves *et al.* (1974) point out, the current problem in rodent control is to overcome the setback caused by the appearance of resistance to the otherwise highly successful anticoagulants, the treatments were done on farms with a history of warfarin resistance.

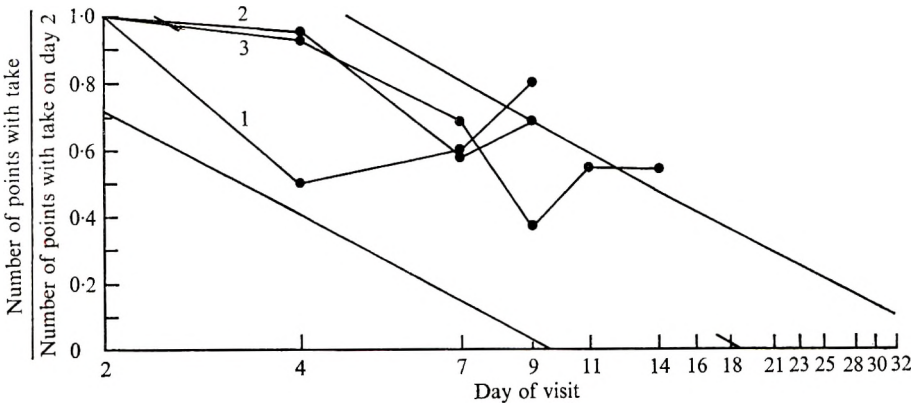

Fig. 1. The results of poison baiting with 0·01 % calciferol plus 0·025 % warfarin on Farms 1–3.

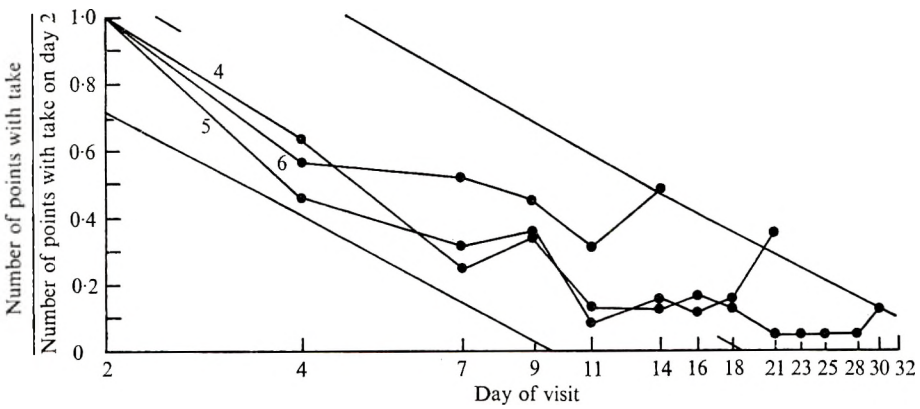

Fig. 2. The results of poison baiting with 0·025 % calciferol plus 0·025 % warfarin on Farms 4–6.

METHODS AND RESULTS

The presence of resistant rats on 17 of the 23 farms that were used was demonstrated by trapping animals on each of the 17 sites at least 4 weeks before the treatments began and subjecting them to the same laboratory test for warfarin resistance that is mentioned by Greaves *et al.* (1974).

The course of each poison treatment was monitored by the method described by Drummond & Rennison (1973) for testing anticoagulant resistance in the field. This essentially involves comparing, on a standard graph, the rate of fall in the number of daily takes of bait with that shown by experience to occur in treatments with 0·025 % warfarin against susceptible populations.

All the farms were surveyed and baiting points selected, and protected wooden bait trays were put in place 2–4 days before baiting commenced. If at the end of a treatment no baits had been touched for 4 days the farm was then resurveyed to make sure that no rats had survived.

Early trials

When the first six farms (1–6) were treated there were few laboratory data available on calciferol plus warfarin to indicate the most likely optimal concentrations at which to use these poisons together in the field. In the event, Farms 1–3 were treated by one pair of operators with 0·01 % calciferol plus 0·025 % warfarin (the normal concentration against *R. norvegicus*) and Farms 4–6 by a second pair of operators with the calciferol at 0·025 %. The six farms were treated simultaneously.

The poison baits were made up by mixing 1 part by weight of a fine oatmeal-based master-mix containing 0·5 % warfarin and either 0·5 or 0·2 % calciferol with 19 parts by weight of 'stabilized' medium oatmeal. Because of the instability of calciferol under damp conditions, all uneaten bait was replaced by freshly prepared material every time the farms were visited (on Mondays, Wednesdays and Fridays) throughout the period of the trials.

The results of the six treatments (1–6) are shown in Figs. 1 and 2. In the case of Farms 1–3 (calciferol at 0·01 %) each of the treatments progressed satisfactorily at first. The poison bait was being accepted well on days 2 and 4 and dead rats were found on all three farms on days 4 and 7 (and day 9 on Farm 1). However on day 7 the bait takes were very much smaller than they had been previously and on the ninth and subsequent days about 70 % of the takes recorded were so small that it was impossible to determine by eye whether anything had been eaten, although the bait had clearly been disturbed by rats. The disturbed baits were nevertheless counted as 'takes' for the purposes of recording the progress of each treatment, for clearly they denoted continuing rat activity.

As Fig. 1 shows, this quickly had the effect of carrying the lines for each of the Treatments 1–3 over the upper bound of the standard graph – a result typical of ordinary warfarin treatments that have failed owing to resistance. It may be concluded that the calciferol made a negligible contribution to the final levels of control obtained (estimated at 20, 32 and 46 %, respectively) and may even have affected them adversely by depressing the appetite of the rats for the poison bait.

In the treatments with 0·025 % calciferol and warfarin (4–6) the number of takes fell steadily over the first 11 days and then the picture (Fig. 2) followed that of Treatments 1–3. The probable interpretation of Fig. 2 is that after day 11 warfarin-resistant rats were recovering from sub-lethal doses of calciferol. Consistent with this is the fact that Treatment 4, which showed the greatest reduction in takes of bait and went on for 31 days, took place on the farm from which was obtained the rat sample with the lowest proportion of resistant animals. The estimated kills on Farms 4, 5 and 6 were 87, 65 and 52 %, respectively.

Trials with calciferol at 0·1 %

Treatments 1 and 6 indicated that if calciferol plus warfarin was going to be effective against the common rat in the field, the concentration of calciferol would need to be increased: and this was confirmed by toxicity data obtained at this stage by Greaves *et al.* (1974). Accordingly in all subsequent treatments with

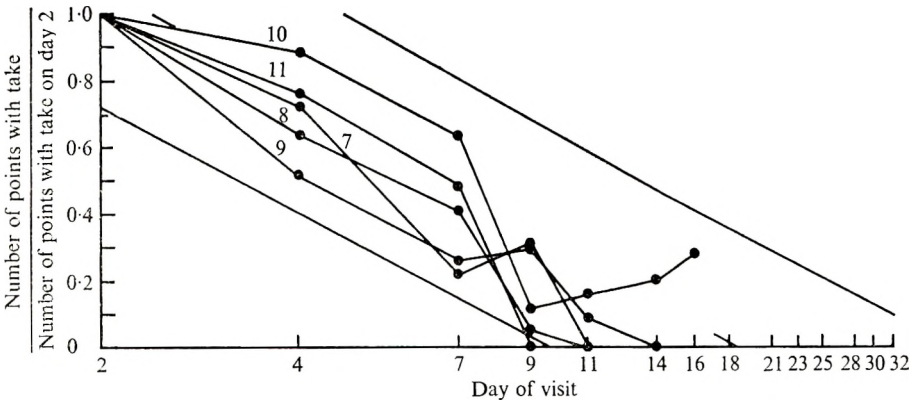

Fig. 3. The results of poison baiting with 0·1 % calciferol plus 0·025 % warfarin on Farms 7–11.

calciferol the concentration used was 0·1 %. At this concentration in the laboratory some rats succumb after 1 day of exposure to the poison and it was feared that in the field the phenomenon of 'poison shyness' as a result of sub-lethal feeding might impair the results of treatments. In the next series of treatments therefore, six were done after 'pre-baiting' with plain bait for 4 days and five, as before, by putting the mixture of 0·1 % calciferol and 0·025 % warfarin down immediately.

The infestation on Farms 7–9 and 12–14 were treated simultaneously and before those on Farms 10 and 11 and 15–17. The two pairs of operators responsible for the work were allocated the method (pre-baiting or direct poisoning) at random so that the team that directly poisoned three of the first six infestations, pre-baited and poisoned three of the second six, while the second team pre-baited on three of the first farms and directly poisoned on two of the second. A sixth direct poisoning treatment that had been planned was not carried out because the pre-treatment trapping was found to have virtually eradicated the rats.

To obtain an even mix of the poison in the bait the best procedure was found to be to mix together 1 part of 0·5 % warfarin in fine oatmeal and 1 part of a 2 % solution of calciferol in an edible oil that by now had become available and then slowly add to this 18 parts of stabilized medium oatmeal. As in the earlier trials the poison bait was replaced by fresh bait at each visit but it has since been ascertained that calciferol in an oil formulation does not deteriorate during the course of a treatment of normal length (M. R. Hadler, personal communication). For the pre-baiting, stabilized medium oatmeal mixed with 5 % maize oil was chosen.

The results of the five treatments in which direct poisoning was used are shown in Fig. 3 and those of the six that followed pre-baiting in Fig. 4. At all eleven sites the considerable 'takes' of poison bait recorded at the first inspection indicated that it had been readily accepted, but thereafter the quantities taken were small.

Dead rats were found only intermittently from the 3rd to the 8th day where poisoning followed pre-baiting, but at every visit from the 4th to the 9th day on

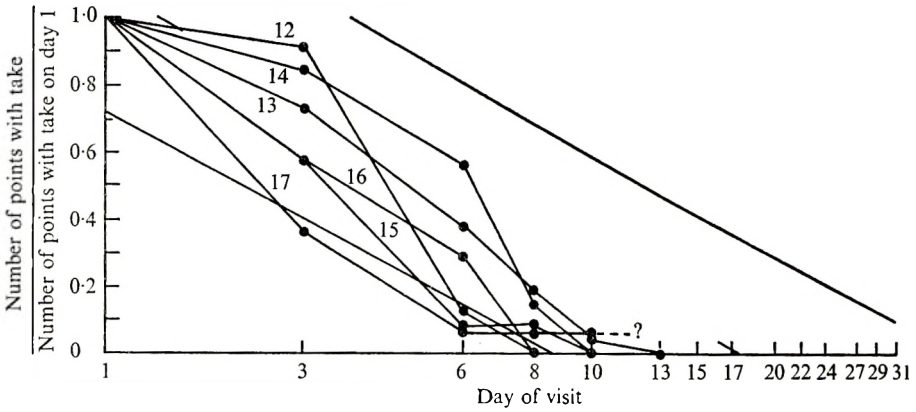

Fig. 4. The results of poison baiting with 0·1 % calciferol plus 0·025 % warfarin after pre-baiting on Farms 12–17. The poison baits taken were counted for the first time after 1 day instead of the usual 2 days in these treatments.

each of the other farms. This suggests that pre-baiting induced more rapid feeding with the result that the majority of rats became moribund within the first 24 hr. of the treatment and died under cover.

Fig. 3 shows that four of the five direct treatments were completely successful. On Farm 10 however an estimated minimum of 28 % of the population was surviving on day 16, when it was decided that no further success would be achieved and the baiting was temporarily discontinued. Beginning 2 weeks later, Farm 10 was pre-baited with dry, cracked wheat. At the end of 9 days three 200 g. takes and seven takes between 50 and 200 g. were recorded but when calciferol plus warfarin was then put down again for 14 days only three or four baiting points were found to have been disturbed at each visit. The only satisfactory explanation of these observations stems from the fact that the size of the rat population on Farm 10 was misjudged before the original treatment and on the first inspection after poison was laid several baits in the area that subsequently gave trouble were found to have been completely eaten. This inadvertent underbaiting might have resulted in a number of animals acquiring sub-lethal doses and developing shyness to one or both of the poisons present in the bait.

Similarly, in the six treatments following pre-baiting (Fig. 4), five were successfully concluded within 14 days. The sixth (Farm 17) fell short of completeness only because rats in a single burrow in a disused deep litter poultry shed regularly disturbed the nearby bait by throwing litter over it, but took neither the pre-bait nor the poison. Obviously this failure could not be attributed to the poison.

As a perhaps more satisfactory check of the effectiveness of calciferol against warfarin-resistant populations of *R. norvegicus* a further six farms (18–23) with a history of resistant rats were selected and treated first with warfarin at 0·025 % and the course of each treatment plotted on the standard graph. Figs. 5 and 6 show that within 7–18 days it could be concluded that five of the six treatments had been unsuccessful because of resistance to the poison, which was therefore removed. The sixth treatment on Farm 20 was more successful but even here it was apparent by day 14 that a few resistant rats were probably present, although

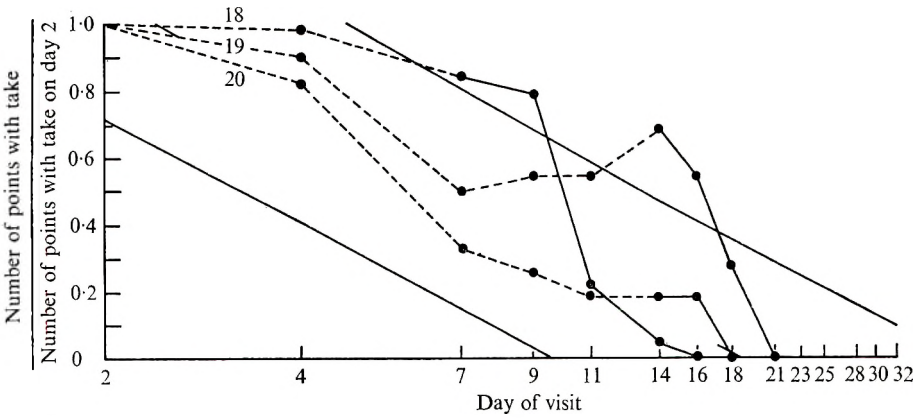

Fig. 5. The results of first poison baiting with 0·025 % warfarin (broken lines) and then adding 0·1 % calciferol to the poison bait (solid lines) on Farms 18–20.

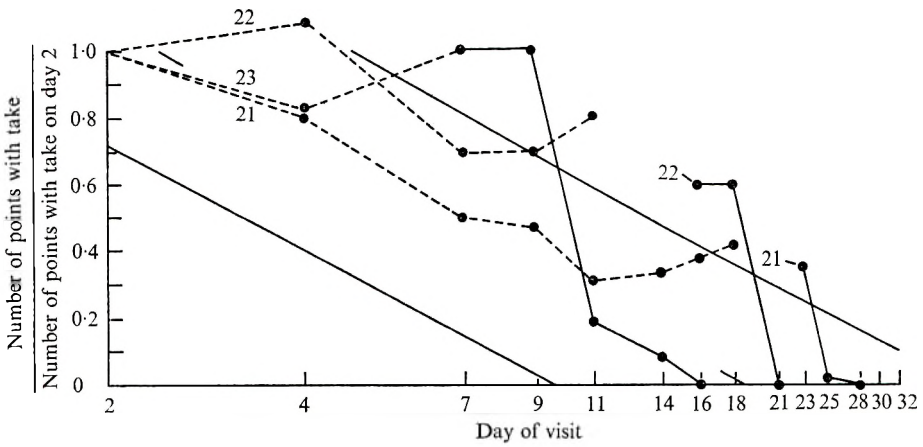

Fig. 6. The results of first poison baiting with 0·025 % warfarin (broken lines) and then with 0·1 % calciferol (solid lines) on Farms 21–23.

it might have taken a rather longer period of baiting to prove it. The poison was therefore picked up on this farm too. On all six farms, immediately or within 2 days (determined by the need, for labour reasons, to synchronize the visits) treatments were then resumed, either with 0·1 % calciferol plus warfarin (Farms 18, 19 and 20: Fig. 5) or with 0·1 % calciferol alone (Farms 21, 22 and 23: Fig. 6). Figs. 5 and 6 show that within 2–9 days the change of poison led to the eradication of the rats on all six sites and this was confirmed by observations continued over several more days. The rapidity with which control was achieved on Farm 20 can be attributed to the small size of the residual infestation and the ease with which it could be baited.

It should be recorded that in some of the treatments done in frosty weather a number of dead house sparrows and hedge sparrows were found in the vicinity of baiting points, in spite of the fact that every precaution had been taken to protect the bait from interference by non-target animals. A similar occurrence has been recorded in trials of calciferol in Scotland (C. M. Boyle, personal communication).

CONCLUSIONS

The effectiveness of the anticoagulant poisons such as warfarin derives from their cumulative effect, and because they do not induce symptoms that inhibit feeding on bait until a lethal dose has been ingested. The above field trials were begun in the belief that calciferol could be used at such a concentration that the same advantages might accrue. In fact, at 0·1 % below which it proved to be ineffective (Treatments 1–6), calciferol seems to behave almost as a single-dose poison. There are indications that it fairly quickly reduces appetite and there is the possibility also that it may induce poison shyness. There is no proof of this, but it is not easy to find another explanation for the results obtained on Farm 10.

In either case, it is suggested that when calciferol is being used, with or without warfarin, the aim should be to encourage the rodents at risk to feed freely from the poison bait from the beginning. A surplus of bait must therefore be available and, in some situations, ensuring this may involve either test-baiting to discover more about the size and distribution of the rat population to be treated or – more effectively still – pre-baiting.

An alternative approach is to begin treatments with warfarin and then follow up with 0·1 % calciferol, thus reducing the risk to non-target animals and using less of the more expensive poison. This method was very successful on Farms 18–23.

In only 3 of the 23 treatments was calciferol used without warfarin, but there seems little doubt that it is effective alone. In fact it is likely that when it is combined with warfarin what happens is that the latter at the most only accounts, or helps to account, for the more warfarin-susceptible individuals while the more warfarin-resistant animals die of calciferol poisoning before a lethal amount of warfarin has been acquired.

The author is indebted to Mr M. R. Hadler of Sorex (London) Ltd who arranged the supply of master-mixes that were used. Thanks are also due to Messrs G. Long, E. Jones and E. Pugh who, led by Mr F. Pritchard, carried out the treatments with great care, often under trying conditions; also to Messrs E. J. Wilson and N. J. Wallace for assistance in the field.

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Lesions experimentally produced by fungi implicated in extrinsic allergic alveolitis

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SUMMARY

The ability of *Aureobasidium pullulans*, *Cladosporium herbarum* and *Cryptostroma corticale* to produce local lesions in the rabbit was examined. Both *C. corticale* and *A. pullulans* can survive *in vivo* and produce the inflammatory response typical of mycetoma. *C. herbarum* failed to grow or survive *in vivo*.

INTRODUCTION

The disease condition of extrinsic allergic alveolitis can be caused by several organisms, including both fungi and actinomycetes (Salvaggio & Buechner, 1971; Pepys, 1969). The actinomycetes which have been implicated, namely, *Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari* have been shown to be capable of causing local mycetoma (Stretton & Bulman, 1974).

Cladosporium herbarum and *Aureobasidium* (*Pullularia*) *pullulans* are common airborne fungi which occur frequently on plants and other objects (Bernstein & Feinberg, 1942; Gregory & Lacey, 1963). Though *Aureobasidium pullulans* has been implicated by Cohen, Merigan, Kosek & Eldridge (1967) in sequoiosis, and *C. herbarum* has been associated by Bernton & Thorn (1937) and Tomsikova, Dura & Novackova (1973) with causing allergic syndromes, neither organism has been considered to be capable of causing local infection. Wynne & Gott (1956) have reported the isolation of *A. pullulans* from granulomas in patients with Hodgkin's disease and it has also been isolated from patients with rheumatoid arthritis (Arthritis & Rheumatism Council, 1966). However, it has been suggested by Emmons, Binford & Utz (1970) that *A. pullulans* can be equated with *Cladosporium werneckii*, an epidermal pathogen, although Cooke (1959) accepted them as different species.

Cryptostroma corticale is the causal agent of maple-bark disease (Towey, Sweany & Huron, 1932) and the organism was obtained by Emanuel, Wenzel & Lawton (1966) from the granulomatous nodules by lung biopsy.

This study examines the ability of these organisms as hyphae or spores to survive and produce local lesions, apart from the granulomatous nodules which occur in the lung.

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MATERIALS AND METHODS

Organisms

Cryptostroma corticale was isolated from infected maple bark, kindly provided by F. J. Wenzel, Marshefield Clinic, U.S.A. *Aureobasidium pullulans* CMI2456 (isolated originally from lymph node mycetoma) and *Cladosporium herbarum* CMI131128 were used throughout.

Media

The organisms were maintained on a medium containing (g./l.) mycological peptone, 10; and dextrose, 40. Solidified media were prepared by the addition of Oxoid Agar no. 3, 1·5 %. The ability to grow on this medium was examined at 28° and 37° C.

Morphological characteristics

The organisms were examined for purity, including the presence of contaminants, before injection and after isolation from experimentally infected animals. *Cryptostroma corticale* was examined according to the description of Emanuel, Wenzel & Lawton (1966), *A. pullulans* according to the description of Wynne & Gott (1956) and *C. herbarum* by its morphological character. They were also examined for haemolytic activity on the nutrient medium with an added 5 % v/v defibrinated horse blood.

Preparation of suspensions

Two ml. of a spore suspension, containing 10^6 spores/ml., were inoculated into 200 ml. of liquid medium for the production of the hyphal form and all organisms were incubated for 48 hr. at 28° C. The hyphae were removed by centrifugation at 4,000 ***g*** and washed 4 times with sterile normal saline (0·9 % w/v) solution. On examination by phase-contrast microscopy these suspensions were free from spores.

Spore suspensions were prepared after inoculation on the surface of 200 ml. of solidified medium in Roux flasks and incubating for 6 days at 28° C. The spores were removed from the surface of the agar by washing with sterile normal saline and then depositing the spores by centrifugation at 4,000 ***g*** and washing 4 times with sterile normal saline.

The deposited cells were resuspended in sterile normal saline using a glass tissue homogenizer (Jencons (Scientific) Ltd., Herts) and the cell density adjusted to give *ca.* 10^2 spores/ml., or 10^2 hyphal fragments/ml. using a Thoma counting chamber. The cell density was also checked to be of the same order in each case by measuring the optical density at 420 nm. (SP500, Pye Unicam, Cambridge). The number of viable units was also checked by carrying out pour-plate counts before injection; in all cases this was *ca.* 10^2/ml.

The suspensions were also plated out on the solidified medium and were found to be free from organisms other than the required fungi.

Suspensions of killed cells were prepared by dividing the above suspensions and heating in an autoclave at 120° C. for 20 min. The absence of viable organisms

from these suspensions was checked by streaking out and by the inoculation of 1 ml. of the suspension into nutrient medium and incubating at 28° C.

Experimental animals and injection procedure

Male Dutch rabbits weighing $2{\cdot}0 \pm 0{\cdot}4$ kg. were used throughout. When the intramuscular (i.m.) route was employed 0·3 ml. of the required suspension was injected into the flank and for the subcutaneous (s.c.) route, 0·2 ml. The rectal temperature was measured daily after injection. Rabbits were killed at intervals of 7 and 14 days after injection.

After necropsy samples of kidney, liver, spleen and any obviously infected tissue were inoculated on nutrient medium and incubated aerobically. Also, representative samples of tissue, including obvious lesions, were excised, fixed immediately in mercuric chloride-formalin solution, dehydrated and embedded in paraffin. Sections of 5 μm. thickness were cut and stained with haematoxylin and eosin, Gram stain, periodic acid Schiff (PAS) and for calcification by the alizarin red S method (Pearse, 1972). Smears prepared from the pus were also examined immediately after necropsy using the PAS stain.

Samples of the pus were checked, by streaking out on the maintenance medium and nutrient agar, for the presence of organisms other than the appropriate organism.

RESULTS

All the organisms grew well on the medium described at 28° C. *C. corticale* grew slowly at 37°. *Aureobasidium pullulans* gave very scanty growth at 37° and *C. herbarum* failed to grow at all at 37°. There was no detectable haemolysis, before or after isolation, of any organism from the rabbit.

There was no significant alteration in the body temperature following injection of any of the organisms.

In the case of *C. corticale* and *A. pullulans* nodules were detected on necropsy when either hyphae or spores had been injected. There were no nodules present when *C. herbarum* was implicated.

The nodules produced by *A. pullulans* were always small (*ca.* 0·5 cm.) and located at the site of injection, typically in the m. semimembranosus (Pl. 1, fig. 1). The periphery of the nodule was avascular.

C. corticale produced larger nodules, *ca.* 3 cm. diameter, located typically on the m. semimembranosus (fig. 2). The periphery of the nodule was marked by an increased vascularity. The pus obtained from the nodule was pale yellow in colour, of a cream-like texture, and contained hyphae. Nodules were produced when either hyphae or spores were injected in 80 % of the animals, by the s.c. or i.m. routes.

In no case were the lesions seen to be spreading, and the nodules were all located at or near the site of initial infection. The *post mortem* examination showed no nodule present on the liver, spleen or kidney, nor were any of the organisms recovered from these organs.

In all cases where the killed cells were injected there was no reaction at the site

of injection nor was there any development of nodules, even at the site of the injection.

There were no organisms present in the samples of pus removed from the nodules other than those initially injected, as judged by their morphology and colonial appearance. Viable organisms were recovered from the nodules produced by *A. pullulans* and *C. corticale;* this included those occurring just at the site of injection. The pus from *C. corticale* contained *ca.* 100 viable units/ml. and *A. pullulans* 50/ml.

The histopathological examination revealed the presence of abscesses showing a typical inflammatory response. The type of abscess produced was similar when either *A. pullulans* or *C. corticale* was the cause (Pl. 1, fig. 3 and Pl. 2, fig. 4). There was evidence of necrosis of muscle fibres, particularly in the case of *C. corticale*, and fibrosis was noted around the periphery of the nodule (figs. 3 and 4). The organisms which produced the limited infections were present predominantly as hyphae, even after the injection of spore suspensions. In the nodules of *C. corticale* some spores remained ungerminated (fig. 5) and normal hyphae could be detected. There were fewer and shorter hyphae present when *A. pullulans* was seen in nodules (fig. 6). There was no evidence of calcification in any of the nodules, even after 14 days.

No organisms were detected in any of the organs examined histopathologically, only in the nodules themselves. There were no histological changes at the site of injection when killed cells had been injected.

DISCUSSION

The ability of *C. corticale* and *A. pullulans* to produce local infections was somewhat unexpected in view of their apparent difficulty in growing at 37° C. *in vitro*. In fact Emanuel *et al.* (1966) stated that *C. corticale* did not grow at 37° C. We found that, although growth was slow, it did occur and subsequently F. J. Wenzel (personal communication) has confirmed this observation. The observation that *A. pullulans* and the plant pathogen *C. corticale* can produce local infection supports the evidence of Lie-Kian-Joe, Tjoei Eng, Kertopati & Emmons (1957) that a plant pathogen *Cercospora apii* can also cause mycetoma of the thigh and thorax as well as being the causal agent of leaf-spot disease.

The lack of growth of *C. herbarum*, *in vivo*, was not surprising in view of its lack of growth at 37° C. *in vitro*, and in this work it served as a suitable control representing a commonly saprophytic group with species which are occasionally pathogenic (Emmons *et al.* 1970).

Both *C. corticale* and *A. pullulans* can survive *in vivo*. The presence of hyphae in the nodules following injection of spore suspensions suggests that limited growth and some differentiation had taken place. Nodules similar to those produced in this instance can be produced by the injection of foreign material which gives a non-specific inflammatory response. However, this is unlikely to have happened, as the injection of heat-killed cells failed to produce nodules or elicit any response, as seen by histological examination.

The organisms did not appear to produce any tissue-destroying enzymes, as judged from the histopathological examination. They were certainly deficient in haemolysin. The tissue destruction which would be necessary to allow even limited spreading could come from a release of proteolytic enzymes from the degranulating leucocytes (Cline, 1970) which are present around the periphery of the nodule.

The spread of infection is limited by fibrosis around the nodule, and similar fibrosis is common in cases of deposition of agricultural dusts in the lung (Rankin, Kobayashi, Barbee & Dickie, 1965). A feature of the nodules produced by these organisms is the absence of calcification, which in other infections may help to limit the spread of the infecting organisms.

The low numbers of organisms present in the nodule are not unexpected in view of the difficulty frequently encountered in obtaining the causal organism from such a lesion (Emmons *et al.* 1970). The pleomorphism exhibited in the case of *A. pullulans* is typical of several fungi where the form seen *in vivo* can differ significantly from that seen *in vitro*. This is seen in sporotrichosis (Howard, 1961) or in actinomycotic mycetoma (Georg *et al.* 1972; Stretton & Bulman, 1974).

With the organisms being present in large numbers in certain environments (Gregory & Lacey, 1963) and *A. pullulans*, in particular, being ubiquitous, the role of these fungi as possible opportunistic invaders may need reappraisal. Emmons (1962) considered the situation of several pathogenic fungi and concluded that so long as the environmental conditions were favourable they were vigorous and self-sufficient saprophytes and were parasites by accident. This is probably true of saprophytic fungi which are opportunistic invaders.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Nodules produced after 7 days following i.m. infection with *Aureobasidium pullulans* as a spore suspension located on the m. semimembranosus. × 1.

Fig. 2. Nodule produced after 7 days following i.m. infection with *Cryptostroma corticale* as a spore suspension, located on the m. semimembranosus. × 0·5.

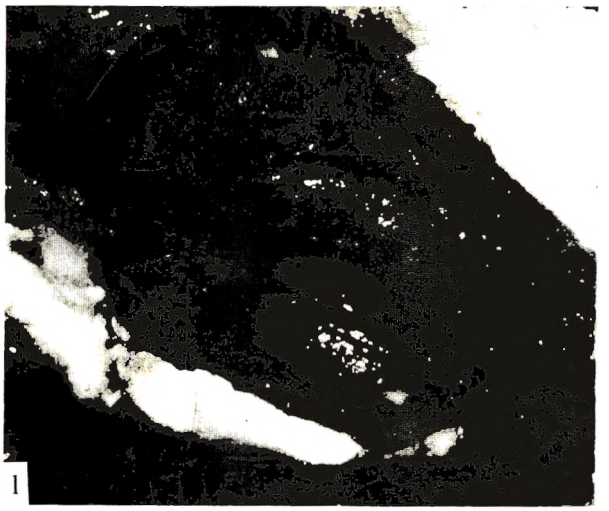
Fig. 3. Nodule produced by *A. pullulans* after 7 days in the m. semimembranosus showing necrosis of muscle fibres and limited fibrosis. H. & E. × 400.

PLATE 2

Fig. 4. Nodule produced by *C. corticale* after 7 days in the m. semimembranosus showing necrotic muscle fibres, fibrosis and inflammatory response. H. & E. × 160.

Fig. 5. Hyphae and spores present in nodule produced by *C. corticale*. PAS, × 1000.

Fig. 6. Short hyphae present in nodule produced by *A. pullulans*. Gram × 1000.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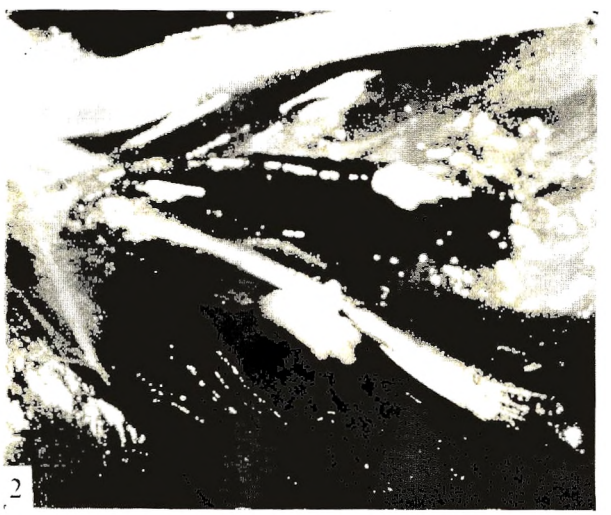
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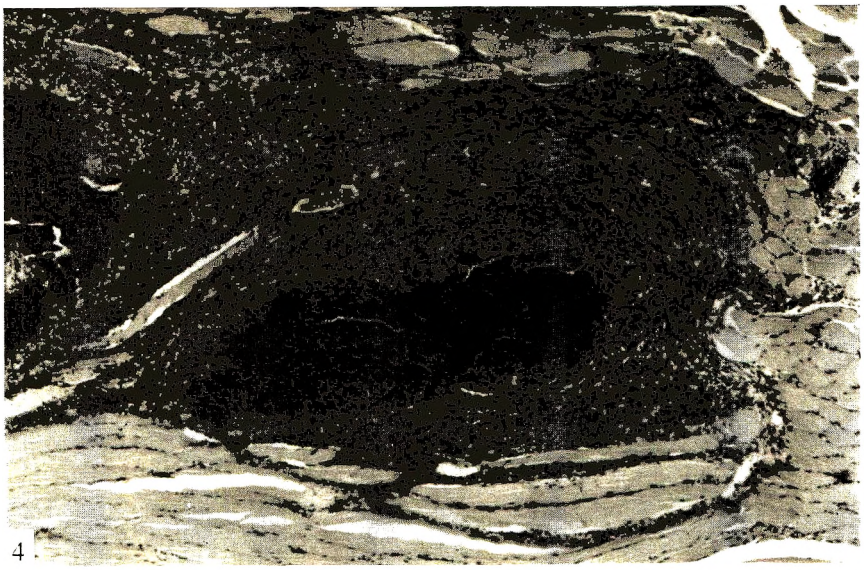

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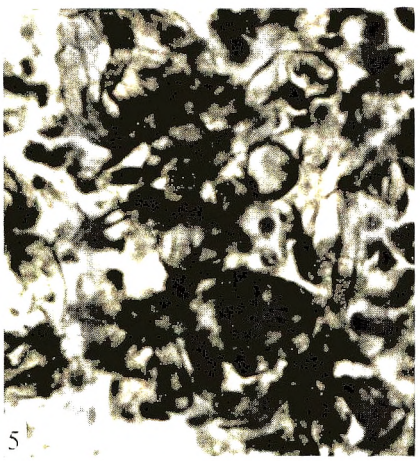

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Phage types of *Staphylococcus aureus* in one hospital 1961–72

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SUMMARY

Between 1961 and 1972, 4547 independent strains of *Staphylococcus aureus* isolated from one hospital were examined for phage type. After 1967 there was a decline in the number of strains received, which we consider reflects a decline in the number of infections in the hospital, and which was largely accounted for by a great reduction in the number of strains in four 'epidemic' types. Overall, the number of multiple-resistant staphylococci received also declined; this was in part due to the decline in the epidemic types and in part to a reduction in the proportion of multiple-resistant strains of all types.

INTRODUCTION

From 1961 to 1972 strains of *Staphylococcus aureus* isolated from in-patients at St Mary's Hospital, London, were routinely tested for phage-type. This paper summarizes the change in phage-type frequencies over the years and reports, in addition, the antibiotic sensitivities for strains examined between 1964 and 1972.

MATERIAL

We made it a rule that all strains of *Staphylococcus aureus* isolated in our diagnostic laboratory from in-patients were to be tested for phage type and we believe that cultures were taken from practically all lesions in which any infection was suspected. Doubtless some strains were missed and others failed to survive, but we know of no systematic changes occurring over the 12 years that would affect the selection of strains for testing. The number of staphylococcal cultures is therefore an index of the number of cases of staphylococcal infection. We have not been able to distinguish infections acquired in hospital from those already present when the patient was admitted. This analysis is based on strains from blood cultures, wounds, minor septic lesions and urines; it includes only one strain of any one phage type from any one patient.

Phage typing was performed by methods, and with phages, provided by the PHLS Staphylococcus Reference Laboratory, Colindale. All the strains were tested at R.T.D. and untypable strains were retested at 1000 × R.T.D. (from 1961

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Table 1. *Phage types of strains* 1961–72

Phage group	Phage pattern	Number of strains
I	80/81	123
	80; 80/+; 81; 81/+	326
	52/52A/80/81; 52/52A/80/+; 52/52A/81/+; 52A/80/81/+	173
	29/52/80; 29/52/+; 29/80/+	286
	29	144
	52A/79; 52A/+; 79/+	164
	Other patterns	318
II	55; 55/+	53
	3C/55/71; 55/71 (+)	75
	Other patterns	223
III	6/7/47/54/75, incl. strains with 3 or more of these reactions	206
	83A; 83A/+	82
	84/85; 84; 85; 84/85/88	269
	77; 77/+	99
	Other patterns	643
Mixed group patterns		416
Untypable		947
Total		4547

Note. No other phage pattern or group of closely related patterns was represented by 50 or more strains.

to 1970) or at 100 × R.T.D. (1971–2); phages 83A, 84 and 85 were not included in the 'concentrated-phage' set. Phages 84 and 85 were not in use during 1961 and 1962, but we have some evidence that strains that would have been lysed by these phages were not then present in more than very small numbers.

The phage types were classified as shown in Table 1. Our definition of type 80/81 is narrower than that used by Parker *et al.* (1974) because preliminary studies showed a striking variation in the prevalence of the type as narrowly defined. Some of the strains classified by Parker *et al.* in the 52, 52A, 80, 81 complex are here included under 'other patterns' in Group I. We have also separated some other types in Group I that we encountered frequently.

Antibiotic sensitivity was determined by the diagnostic bacteriology laboratory using Oxoid 'Multodisks'. Penicillin, tetracycline, chloramphenicol, neomycin, erythromycin and cloxacillin were used throughout; streptomycin was included until 1967; fucidin and cephaloridine were introduced in 1968.

RESULTS

We had available for analysis the phage-typing results of 4547 'independent' strains of *Staphylococcus aureus*. The number received each year fluctuated between about 375 and 500 from 1961 to 1967 and subsequently declined (Fig. 1). As

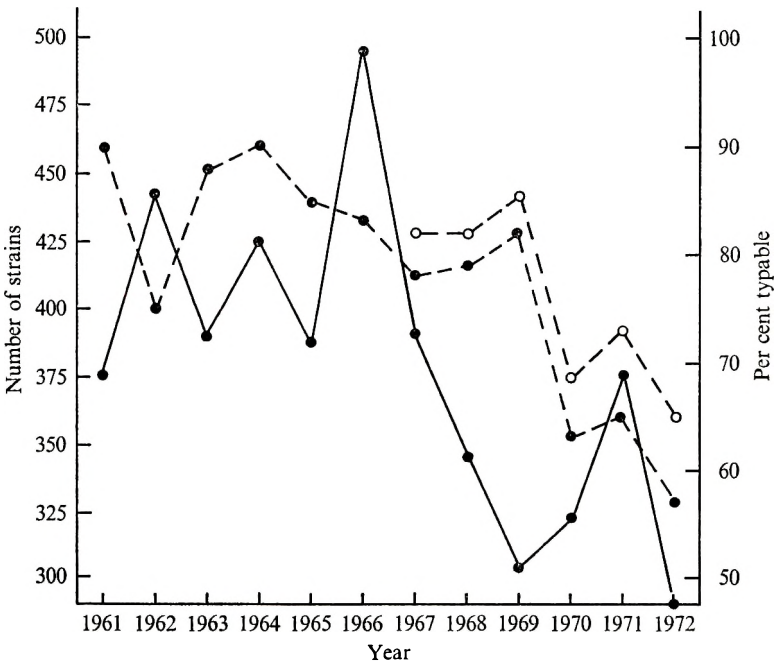

Fig. 1. Number of strains received 1961–72. ●—●, total; ●---●, per cent typable; ○---○, per cent found typable at Colindale.

already noted we believe that this decline reflects a decline in the number of staphylococcal infections recognized in the hospital.

From 1961 to 1969 the proportion of strains that were typable remained at about 80 % or more, but in 1970–2 the percentage typable was much lower (Fig. 1). From 1967 to 1972 we contributed a random sample of 100 strains per year to Dr M. T. Parker's survey (Parker *et al.* 1974) and the results of retesting these strains at Colindale show that the decline was not due simply to technical difficulties in our laboratory; at Colindale only 65 % of the 100 strains examined in 1972 proved typable, compared with our figure of 57 % for the 290 strains typed at St Mary's.

A total of 12 different phage 'types' were each represented by 50 or more strains (Table 1) and 2000 (44·0 %) of the total 4547 strains fell into one or other of these, which may be called 'endemic' types; 1600 strains fell into types each represented by only a small number of strains.

The 12 endemic phage types seemed to fall into two groups. Four types (80/81, 83A, 6/7/47/54/75 and 84/85) were very prevalent during the early years (constituting 23 % of all strains tested in 1961–4) and then declined (Fig. 2). These are all types that have been widely recognized elsewhere as responsible for 'epidemics' of hospital infection, and may for convenience be termed 'epidemic' types.

The remaining 8 endemic types showed substantial variations in number from year to year but secular trends were not obvious (Fig. 3).

The changes in the distribution over the three groups, epidemic, other endemic

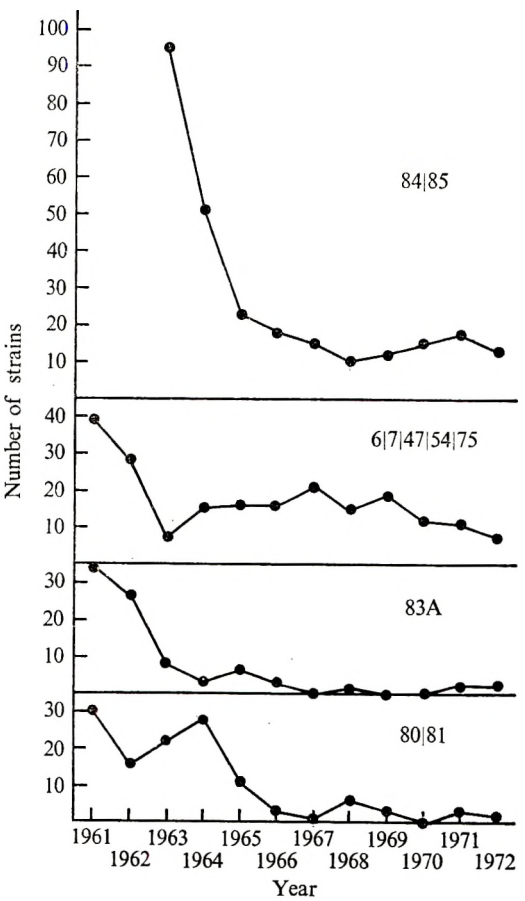

Fig. 2

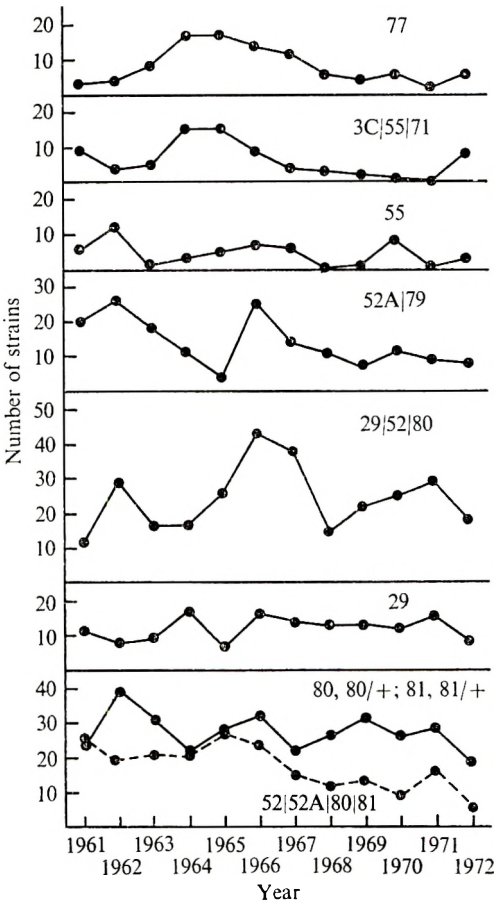

Fig. 3

Fig. 2. Changes in prevalence of epidemic types.

Fig. 3. Changes in prevalence of non-epidemic endemic types.

and sporadic are illustrated by smoothed curves drawn from 3-year moving averages in Fig. 4. There was a steep decline in the prevalence of the epidemic types between 1964 and 1967, from about 100 per year to about 30 per year. The number of strains of the other endemic types showed, over the whole period, a slight decline, but the numbers of 'other types' and untypable strains showed, if anything, a slight increase from just under to rather over 200 per year. The decline in the total number of strains received is, therefore, accounted for very largely by the decline in frequency of strains in the epidemic and other endemic phage types. In so far as 'strains received' may be equated to 'infections' this appears to indicate a decline in the incidence of infections due to the types that have commonly been associated with epidemics of hospital infection, but little change in the infections due to the great number of sporadic types. Many of these doubtless represent self-infection with staphylococci carried before admission to hospital, or sporadic cross-infections from staff.

Records of antibiotic sensitivity tests were available for almost all the strains

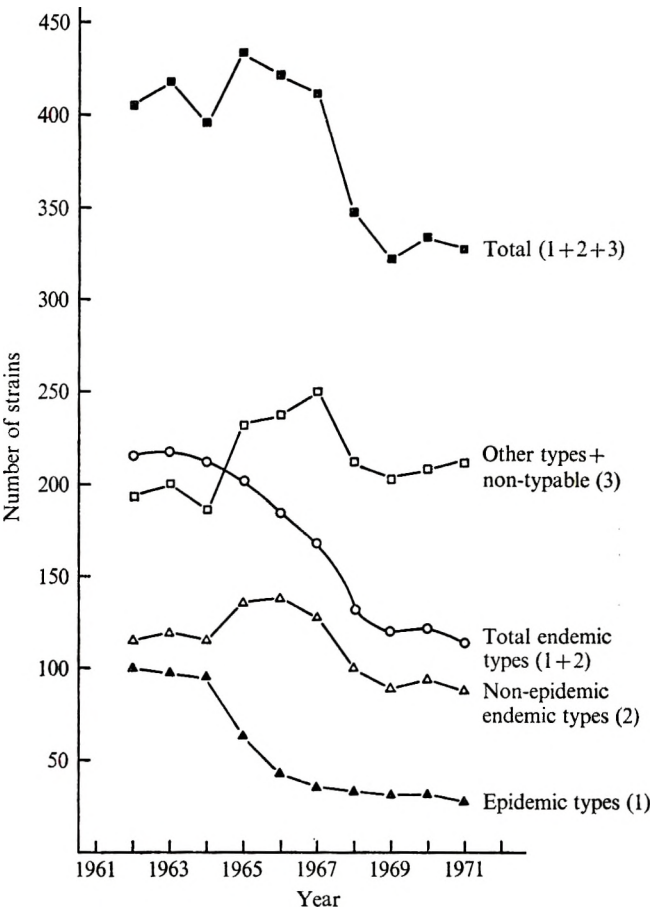

Fig. 4. Prevalence of staphylococci in epidemic, endemic and other types: 3-year moving average, 1962–71.

received from 1964 to 1972. Strains were classed as (S) sensitive to all antibiotics tested, (P) resistant to penicillin but to no other drug, or (M) resistant to penicillin and others, or resistant to other drugs while sensitive to penicillin (multiple-resistant). The year-by-year trend for all strains tested is shown in Fig. 5. There was no consistent variation for the number of sensitive strains received but there was a clear decrease in the number of multiple-resistant strains. After 1965 penicillin-resistant strains were commoner than in 1964 and 1965. The decrease in the number of multiple-resistant strains is clearly associated with, although not fully explained by, the decrease in the number of strains of the endemic types, especially the epidemic types (Fig. 6). Over the 9 years as a whole, 67 % of the strains in the epidemic types were multiple-resistant, while 25 % were penicillin-resistant and only 8 % sensitive. There was a slight decline in the number of multiple-resistant strains and a rise in the number of penicillin-resistant strains in the sporadic types (including untypable strains) (Fig. 7).

The number of strains of individual phage types tested in each year was naturally small, but, as seen in Table 2, the proportion of multiple-resistant strains in

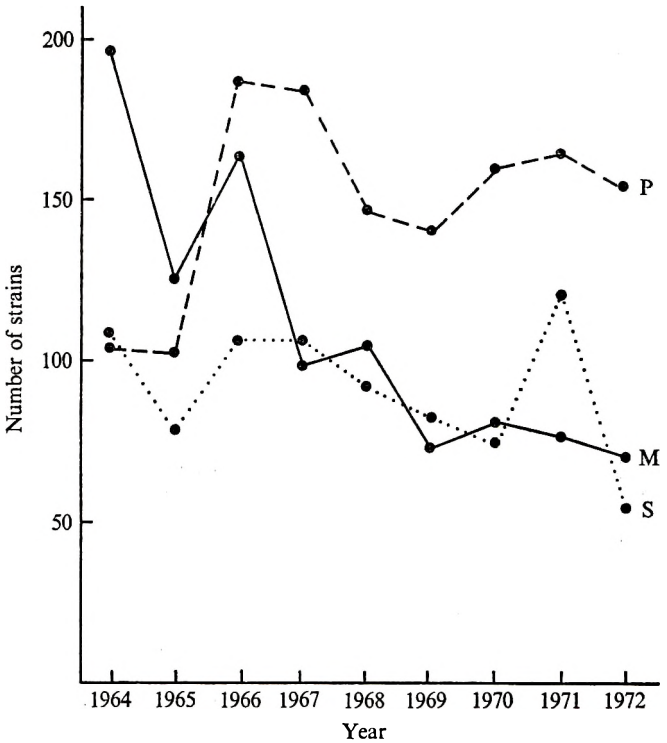

Fig. 5. Prevalence of antibiotic-sensitive (S), penicillin-resistant (P) and multiple-resistant (M) staphylococci, 1964–72.

three of the four epidemic types was lower in 1968–72 than in 1964–7, as was the proportion in other groups of types. The overall decline in the number of resistant strains was therefore only in part due to the disappearance of those types characteristically having a very high proportion of multiple-resistant strains.

DISCUSSION

This report supplements the larger survey reported by Parker *et al.* (1974) within which some of our results were included. Our survey began in 1961 when staphylococcal infection in hospital was a topic of much concern and when 'epidemics' of such infection seemed to be common; by the end of the period, in 1972, epidemics were generally considered to be rare and staphylococcal infections were no longer regarded as so serious a problem.

To the extent that the numbers of staphylococci isolated in our diagnostic laboratory reflect the incidence of infection in the hospital – and we think that they are probably a reasonable indication – St Mary's Hospital has experienced a decline in staphylococcal infection, mostly in the period from 1968 to 1972, and for this reason we have presented the numbers of strains received, rather than the proportion, in various phage-types or resistance categories.

Examination of the trends for a number of phage-types showed immediately that the decline was associated with a striking drop in the numbers of strains in

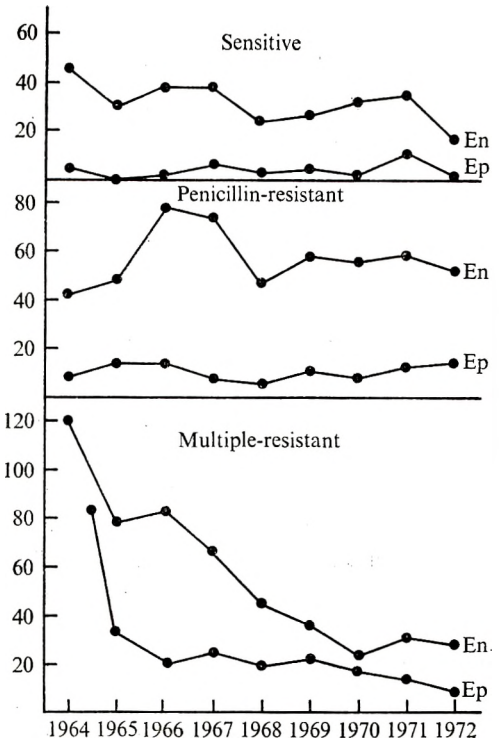

Fig. 6

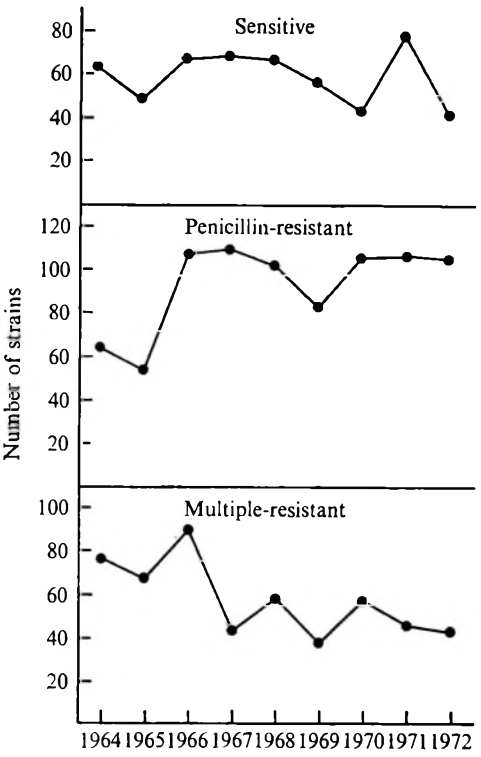

Fig. 7

Fig. 6. Antibiotic sensitivity of strains in epidemic (Ep) and other endemic (En) types.

Fig. 7. Antibiotic sensitivity of strains in sporadic types and of untypable strains.

Table 2. *Proportion of multiple-resistant staphylococci in various groups of phage-types*, 1964–7 *and* 1968–72

		Percentage multiple-resistant	
		1964–7	1968–72
Epidemic types	80/81	90 (41)	79 (14)
	83A	80 (10)	20 (5)
	6/7/47/54/75	34 (61)	46 (59)
	84/85	94 (100)	58 (66)
Total endemic types		45 (715)	29 (565)
Other types and untypable		31 (264)	24 (1015)

Figures in parentheses = number of strains.

four types, 80/81, 6/7/47/54/75, 83A and 84/85. These are all types that have been recognized as causing 'epidemics' in hospitals in many parts of the world. In other analyses there have been indications that particular epidemic types were 'replaced': 80/81 by 52/52A/80/81 and similar strains, and 83A by 84/85 and so on. There is no clear evidence of such replacement in our data for numbers, as opposed to proportions, in particular types. There was a smaller drop in infections due to three of the other seven 'endemic' types, namely 77, 3C/55/71 and 52A/79. About half (46 %) of the strains tested in 1961 fell into one or other of a very large number of phage types, each represented by very few strains, or were untypable; there was no significant decline in infections due to these strains over the 12 years, which therefore increased in relative prevalence and constituted 66 % of the total received in 1972.

The decline in infections may therefore be accounted for by the great decline in the frequency of strains in a few 'epidemic' types.

We have no records of antibiotic sensitivity during the first three years of the survey, but from 1964 onwards there was a decline in the number of infections due to multiple-resistant staphylococci and little change in the frequency of infections due to sensitive strains or strains resistant only to penicillin. In part the decline in multiple-resistant strains is attributable to the decrease in the number of strains in the generally resistant epidemic types, but there was also a decrease in the proportion of multiple-resistant strains in all the groups of strains.

One can only speculate on the cause of these changes. An isolation ward was opened in the hospital in 1961 and was replaced by a larger isolation ward in 1966; these wards were used to segregate patients with overt staphylococcal infections. There was over the years a steady increase in the general 'hygienic' facilities in the hospital and latterly the use of pre-sterilized and disposable materials. We have not had an explicit antibiotic policy. It may be that a progressive increase in the efficiency of our hygienic precautions has hindered the transmission of the multiple-resistant staphylococci and that perhaps, as Parker *et al.* (1974) have suggested, an increase in the range of antibiotics available has altered the selection pressures that formerly operated in favour of the epidemic strains. Or it may be that we are going through a fortunate period when there happen to be no very communicable staphylococci in circulation.

We are grateful to Dr M. T. Parker and Dr Elizabeth Asheshov for the supply of typing phages and for much helpful advice.

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Spread of *Escherichia coli* colonizing newborn babies and their mothers

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SUMMARY

Most babies are colonized by the predominant strains of *Escherichia coli* present in their own mother's faecal flora. Those babies who did not acquire their maternal faecal flora acquired strains of *E. coli* belonging to a small number of the possible serotypes. Moreover, the same serotypes were found in several babies and other mothers, suggesting spread within the ward. These few strains included some of the O groups which had previously commonly been found as urinary pathogens. These strains may have increased potentialities for colonization of human bowel. Antigenic and biochemical variation was observed among the strains.

INTRODUCTION

In previous papers (Bettelheim *et al.* 1974*a*, *b*) it was shown that the majority of babies are colonized with strains of *Escherichia coli* which are present in the maternal faecal flora. There were some babies who did not acquire their maternal strains as far as could be ascertained with the techniques used. The strains were characterized by O and H serotypes, their ability to ferment six carbohydrate substrates and their antibiotic resistance. Using these markers, the spread of the strains from patient to patient was examined.

MATERIALS AND METHODS

The collection of strains and determination of their characteristics has been described in the two previous papers (Bettelheim *et al.* 1974*a*, *b*).

The patient numbers are those used previously. The same abbreviations are used throughout and given in Table 1. In addition to the previous 33 mothers and babies described earlier, a further 4 mothers and their babies who had been delivered by Caesarean section were studied. Their patient numbers are 9, 11, 34 and 36.

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Table 1. *Abbreviations used to distinguish strains of* Escherichia coli

Marker Drug resistance	Abbreviation	Marker Carbohydrates fermented	Abbreviation
Ampicillin	A	Dulcitol	Du
Streptomycin	S	Maltose	Ma
Tetracycline	T	Raffinose	Rf
Chloramphenicol	C	Rhamnose	Rh
Kanamycin	K	Sorbose	Ss
Sulphadimidine	Su	Sucrose	Sc
Nalidixic acid	Nal		
Trimethoprim	Tr		
Gentamicin	G		
Fully sensitive	F/S		

Table 2. *Evidence of spread of* Escherichia coli *amongst babies*

Strain of *E. coli* isolated from stool			Date of isolation	Patient
O3:H2	Su	Du, Ma	22–6 March 1972	2 mother
O3:H2	Su	Du, Ma	24–9 March 1972	2 baby
O3:H2	SSu	Du, Ma	4–6 May 1972	16 baby
O3:H2	SSu	Du, Ma	11–16 May 1972	24 baby
O3:H2	SSu	Du, Ma	18 May 1972	32 baby
O3:H2	SSu	Du, Ma	18–24 May 1972	33 baby
O3:H2	SSu	Du, Ma	21–5 May 1972	36 baby
O162:H10	T	Ma	26 April–2 May 1972	10 mother
O162:H10	T	Ma	28 April–2 May 1972	10 baby
O162:H10	T	Ma	2–3 May 1972	12 baby
O162:H10	T	Ma	13–14 May 1972	23 baby
O162:H10	F/S	Ma	26 April 1972	10 mother
O162:H10	F/S	Ma	28 April 1972	10 baby
O162:H10	F/S	Ma	2–5 May 1972	12 baby
O162:H10	F/S	Ma	13 May 1972	23 baby
O1:H−	F/S	Ma, Ss	7–12 May 1972	26 mother
O1:H−	F/S	Ma, Ss	11–16 May 1972	26 baby
O1:H−	F/S	Ma, Ss	19 May 1972	35 baby
O10:H4	F/S	Ma, Rh	3 May 1972	21 mother
O10:H4	F/S	Ma, Rh	4–5 May 1972	21 baby
O10:H4	F/S	Ma, Rh	11 May 1972	25 baby

RESULTS

In all 2,780 strains of *E. coli* were examined from 37 mothers and their babies. The characterization of similar strains isolated from mothers and their babies has been previously described. The remaining strains were then examined for common characteristics. It was noted that among the remaining strains isolated from mothers' faeces, there was a very large number of sero- and biotypes. However, from the babies' stools only a limited number of types were isolated.

It was found that there were four strains of the same O and H serotypes and biotypes which occurred in several babies (Table 2). The antibiotic resistance of some of these strains was not identical but loss of a resistance to an antibiotic which is plasmid-mediated is well known. Amongst *E. coli* O/R and H+/H−

Table 3. *O/R and H+/H− variation in* Escherichia coli *spread amongst mothers and babies*

Strain of *E. coli* isolated from stool or mucus			Date of isolation	Patient
O6:H1	F/S	Du, Ma, Rh, Ss	1 May 1972	16 mother
O6:H1	F/S	Du, Ma, Rh, Ss	3 May 1972	20 baby
O6:H−	F/S	Du, Ma, Rh, Ss	3 May 1972	20 mother
O6:H1	F/S	Du, Ma, Rh, Ss	6–11 May 1972	22 mother
O6:H1	F/S	Du, Ma, Rh, Ss	11–13 May 1972	22 baby
O2:H4	Su	Du, Ma, Ss	16 May 1972	33 baby
O2:H4	F/S	Du, Ma, Ss	20–24 May 1972	34 mother
O2:H−	A	Du, Ma, Ss	26 May 1972	34 baby
O1:H−	F/S	Du, Ma, Rh, Ss	22–7 March 1972	1 mother
O1:H−	F/S	Du, Ma, Rh, Ss	22–8 March 1972	1 baby
R:H7	F/S	Du, Ma, Rh, Ss	25–8 March 1972	1 baby
R:H7	F/S	Du, Ma, Rh, Ss	26–8 March 1972	3 mother
O1:H7	F/S	Du, Ma, Rh, Ss	5 May 1972	13 mother
R:H7	F/S	Du, Ma, Rh, Ss	5–9 May 1972	15 baby
O1:H−	F/S	Du, Ma, Rh, Ss	17 May 1972	32 mother
R:H7	F/S	Du, Ma, Rf, Rh, Ss	20–3 April 1972	7 mother
O1:H7	T	Du, Ma, Rf, Rh, Ss	2–8 May 1972	19 mother
R:H7	F/S	Du, Ma, Rf, Rh, Ss	2 May 1972	19 mother
O1:H7	F/S	Du, Ma, Rf, Rh, Ss	8 May 1972	19 mother
O1:H−	T	Du, Ma, Rf, Rh, Ss	8 May 1972	19 mother
R:H7	T	Du, Ma, Rf, Rh, Ss	8 May 1972	19 mother
R:H7	F/S	Du, Ma, Rf, Rh, Ss	4–8 May 1972	19 baby

Table 4. *Spread of serotypically related strains of* E. coli *with differing capabilities to ferment carbohydrates, amongst mothers and babies*

Strain of *E. coli* isolated from stool or mucus			Date of isolation	Patient
O18:H14	F/S	Du, Ma, Rf, Rh, Sc	27 March 1972	3 mother
O18:H14	F/S	Du, Ma, Rh	20 April 1972	5 mother
O18:H14	F/S	Du, Ma, Rh	21 April 1972	4 mother
O18:H−	F/S	Ma	2 May 1972	17 mother
O18:H14	F/S	Ma, Rf, Rh, Sc	3 May 1972	21 mother
R:H14	F/S	Ma, Rf, Rh, Sc	3 May 1972	21 mother
O18:H14	F/S	Ma, Rf, Rh, Sc	3–5 May 1972	21 baby
R:H14	F/S	Ma, Rf, Rh, Sc	3–5 May 1972	21 baby
O129:H30	F/S	Du, Ma, Rf, Rh, Sc	27 April 1972	8 mother
O129:H30	F/S	Ma, Rf, Rh, Sc	27 April 1972	8 mother
O129:H30	F/S	Ma	10 May 1972	27 baby
O19:H7	F/S	Ma, Rf, Rh, Sc	26 March 1972	2 mother
O19:H−	F/S	Du, Ma, Rf, Rh, Ss, Sc	22 April 1972	5 baby
O19:H−	F/S	Du, Ma, Rf, Rh, Sc	27 April 1972	8 mother
O19:H7	F/S	Ma	8 May 1972	23 baby
O19:H7	F/S	Du, Ma, Rf, Rh, Sc	10 May 1972	27 baby
O19:H7	F/S	Ma, Rf, Rh, Sc	10 May 1972	27 baby
O19:H−	F/S	Ma, Ss, Sc	11 May 1972	23 mother
O19:H−	F.S	Ma	11 May 1972	23 mother

Table 5. *Complex variations amongst possibly related strains of* E. coli *from mothers and babies*

Strain of *E. coli* isolated from stool or mucus			Date of isolation	Patient
O82:H11	F/S	Du, Ma, Rh, Sc	24 April 1972	8 mother
O82:H31	A	Du, Ma, Rf, Rh	28 April 1972	13 mother
O82:H−	ASSu	Ma, Rh	29 April 1972	9 mother
O82:H−	Su	Ma, Rh	29 April 1972	9 mother
O82:H31	F/S	Du, Ma, Rf, Rh	2–4 May 1972	13 baby
O82:H31	F/S	Du, Ma, Rf, Rh	3 May 1972	9 baby
O106:H−	F/S	Du, Ma, Rh	22 March 1972	3 mother
O106:H34	F/S	Du, Ma, Rh	8 May 1972	24 mother
O106:H7	F/S	Du, Ma, Rh	8 May 1972	24 mother
R:H7	F/S	Du, Ma, Rh	8–11 May 1972	23 mother
R:H7	F/S	Du, Ma, Rh	8 May 1972	23 baby
R:H7	F/S	Du, Ma, Rf, Rh, Sc	8 May 1972	23 baby
O7:H−	F/S	Ma, Rf, Rh, Sc	22 March 1972	2 mother
O7:H4	F/S	Du, Ma, Rh	24 March 1972	3 baby
O7:H4	F/S	Ma, Rh	26–9 March 1972	3 baby
O7:H−	F/S	Ma, Rf, Rh, Ss, Sc	16–17 April 1972	6 mother
O7:H−	F/S	Ma, Rf, Rh, Sc	16–17 April 1972	6 mother
O7:H−	F/S	Ma, Rh	28 April 1972	13 mother
O7:H−	F/S	Ma, Rf, Rh, Ss, Sc	28 April–5 May 1972	13 mother
R:H30	F/S	Ma	29 April 1972	9 mother
O7:H−	F/S	Ma, Rf, Rh, Ss, Sc	2–6 May 1972	13 baby
O7:H30	F/S	Ma	3 May 1972	13 mother
O7:H−	F/S	Ma, Rf, Rh, Sc	3 May 1972	9 baby
NT:H30	F/S	Ma	5 May 1972	13 mother
O7:H−	F/S	Ma, Rf, Rh, Ss, Sc	3–7 May 1972	9 baby
O7:H6	F/S	Ma, Ss, Sc	11 May 1972	23 mother
O7:H−	F/S	Ma, Ss, Sc	20–1 May 1972	36 baby

variation within a strain is an accepted phenomenon and therefore, if other markers are identical, the isolation of pairs of such strains may imply their relatedness. There were three such groups of strains isolated (Table 3) and in addition non-motile variants of strains listed in Table 2 were found.

The presence of tetracycline resistance and raffinose fermentation have been shown to be due to a plasmid (Ørskov & Ørskov, 1973). The strains from mother and baby 19 are therefore included with the other O1:H7 strains. The possibility that the ability to ferment other carbohydrates is plasmid borne caused the linking of strains with identical O and H antigens but differing fermentation ability (Table 4).

In our previous paper (Bettelheim *et al.* 1974*b*) we noted that within certain mother and baby pairs there was some evidence that more complex variation occurred. Table 5 shows the spread of such related strains between different mothers and different babies.

DISCUSSION

In our previous papers (Bettelheim *et al.* 1974*a*, *b*) we showed that the majority of babies were colonized by strains of *E. coli* which were present in their mothers'

faecal flora. A few babies acquired strains not present in their maternal flora. It was noted that many different strains of *E. coli* were isolated from mothers, whereas only a few of these strains were isolated from the babies. Moreover, most of these strains were isolated on several occasions from several mothers and babies. The O groups of these strains were O1, O2, O3, O6, O7, O10, O18, O19, O82, O106, O129 and O162. Five of these twelve O groups are among the nine O groups (O1, O2, O4, O6, O7, O11, O18, O39 and O75) shown by Gruneberg, Leigh & Brumfitt (1968) to occur in urinary tract infections and in relatively large numbers in human faeces. These strains might be more prevalent in the environment or possibly have a different potentiality for colonization of babies. It has been suggested that some animal and human pathogenic strains have such differing potentialities for their hosts (Williams Smith & Halls, 1967).

Having considered in the earlier study that complex variation of sero- and biotypes may occur in the transfer of strains from mothers to their own babies, we feel that this may also occur within the maternity ward.

We are indebted to the Wellcome Trust and to the Board of Governors of St Bartholomew's Hospital for a grant in support of this work. The expert technical assistance of Mrs E. Blackburn is gratefully acknowledged. We would also like to express our thanks to Miss M. Pollack and her staff in the Department of Midwifery for their diligent help in the collection of specimens.

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Immunity to influenza in ferrets

XI. Cross-immunity between A/Hong Kong/68 and A/England/72 viruses: serum antibodies produced by infection or immunization

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SUMMARY

The degree of immunity due to cross-reactions between antibody to influenza virus A/Hong Kong/1/68 and A/England/42/72 was studied in ferrets. Ferrets were immunized with the viruses by either live infection or by inoculation with inactivated virus vaccines. The vaccines were given with Freund's incomplete adjuvant or were given to ferrets previously infected with influenza virus A/PR/8/34. As a result of these immunizations the animals all produced similar titres of serum HI antibody to the immunizing virus, although the degree of cross-reaction with the other virus strain was variable. After immunization the animals were challenged by infection with an A/Eng/42/72-like virus and their degree of immunity was measured. It was found that the greatest immunity was in ferrets previously infected with the homologous A/Eng/42/72 virus. Animals previously infected with A/HK/68 virus also showed a measurable degree of immunity to A/Eng/42/72 infection, and this was greater than that found in animals given inactivated virus vaccines. The immunity produced by the vaccines was approximately equal, regardless of which vaccine or method of immunization was used. Thus, live infection produced a more effective, broader immunity than did the use of inactivated virus vaccines.

INTRODUCTION

The ferret provides a useful model for the study of influenza, since the disease in this animal resembles that of man (Smith, Andrews & Laidlaw, 1933; Haff, Shriver, Engle & Stewart, 1966; Potter *et al.* 1972*a*). After influenza A virus infection, virus can be recovered from nasal washings, and specific antibody is found in both serum and nasal washings. In addition, the infection can cause a febrile reaction and increased concentrations of protein can be found in nasal washings. After the primary infection the animals are completely immune to challenge infection with the homologous virus for 5 weeks or more, as indicated by failure to recover virus from nasal washings, and the absence of further antibody production (Francis & Stuart-Harris, 1938; Potter *et al.* 1972*a*). However, immunization with inactivated virus vaccine did not produce the same degree of immunity to challenge infection as found after infection (Potter, Shore, McLaren

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& Stuart-Harris, 1972*b*). Serum HI antibody was only produced in normal ferrets given the vaccine together with adjuvants (Potter *et al.* 1972*b*) or in ferrets which had been previously infected with a heterotypic influenza A virus (McLaren & Potter, 1974).

The present study examines the degree of cross-immunity between influenza viruses A/Hong Kong/68 and A/England/42/72; this latter virus is a variant of A/HK/68 virus (Schild *et al.* 1973) which caused epidemic infection in England in the winter of 1972–3, although at this time the population had relatively high titres of serum HI antibody to A/HK/68 which cross-reacted with A/Eng/72 virus (Pereira *et al.* 1972). The antibody response of ferrets was studied after infection with either A/HK/68 or A/Eng/72 virus, or after immunization with either A/HK/68 or A/Eng/72 vaccine with Freund's incomplete adjuvant, or by inoculation with vaccine of animals previously infected with influenza virus A/PR/8/34 (McLaren & Potter, 1974). The immunity of the animals was then tested by challenge infection with influenza virus A/Mill Hill/72.

MATERIALS AND METHODS

Viruses and virus vaccines

Influenza viruses A/PR/8/34 (H0N1), A/Hong Kong/1/68 (H3N2), A/England/42/72 (H3N2), A/Mill Hill/1/72 (H3N2) and recombinant virus MRC-2 (A/PR/8/34 × A/Eng/42/72 H3N2) were all obtained from Dr G. C. Schild (World Influenza Centre National Institute for Medical Research, London); the last two strains are antigenically similar to A/Eng/42/72. The viruses were grown in the allantoic cavity of hen's eggs incubated for 48–72 hr. at 33° C.

Inactivated A/Aichi/68 virus vaccine containing 300 CCA/ml. was kindly supplied by Dr Hennessen (Behringwerke AG, Marburg Lahn). Formalin/β-propiolactone inactivated A/Eng/42/72 virus vaccine, containing 600 i.u./ml., was obtained from BDH Pharmaceuticals Ltd., London.

Experimental design

Groups of ferrets were infected intranasally with approximately 10^6 EID 50 of either A/Eng/72 or A/HK/68 virus; infection was confirmed by re-isolation of virus from nasal washings collected 3 days after infection and by the production of serum HI antibody. Groups of ferrets, which had been infected with A/PR/8/34 virus 7 weeks previously, were inoculated intramuscularly with a 0·5 ml. volume of either 300 CCA of inactivated A/Aichi/68 virus vaccine or with 300 i.u. of inactivated A/Eng/72 virus vaccine. Further groups of normal ferrets were inoculated with the same doses of vaccine mixed with an equal volume of Freund's incomplete adjuvant.

Eight to nine weeks after the A/Eng/72 or A/HK/68 infection, or 5 weeks after immunization with the inactivated vaccines, all the ferrets, together with a group of normal ferrets, were challenged by intranasal infection with approximately $4 \times 10^{7.0}$ EID 50 of influenza virus A/Mill Hill/1/72. Nasal washings for virus isolation were collected on the 2nd day after infection, and nasal washings for

protein and antibody measurements on day 3 and subsequent alternate days, as described previously (Potter *et al.* 1972*a*). Serum specimens were collected from each ferret before and 15 days after challenge infection.

Virus isolation

The isolation of virus from nasal washings was carried out as described previously (Potter *et al.* 1972*a*).

Protein estimation

The protein concentration of 10-fold concentrated nasal washings was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Haemagglutination inhibition (HI) tests

HI tests on sera and concentrated nasal washings were carried out as described previously (Potter *et al.* 1972*a*), but with an interval of approximately 60 min between the addition of virus and fowl erythrocytes. Recombinant virus MRC-2 was used in HI tests for antibody to A/Eng/42/72 since this virus was less sensitive than the other strains of this virus to the non-specific inhibitors present in ferret sera.

RESULTS

Serum antibody response of ferrets to infection or immunization with influenza viruses

Sera collected from ferrets 8–9 weeks after infection with either A/Eng/72 or A/HK/68 virus showed high titres of HI antibody to both viruses (Table 1). Titres were usually highest for the homologous infecting virus, and the degree of cross-reaction was variable. Thus, for ferrets infected with A/Eng/72 virus, the serum HI titres were higher to the homologous virus in three animals, but higher to the heterotypic A/HK/68 virus in one ferret (Table 1). For ferrets infected with A/HK/68 virus homologous and heterotypic serum HI antibody titres were similar (Table 1).

Ferrets infected with influenza virus A/PR/8/34 and then immunized 7 weeks later with 300 CCA of A/HK/68 vaccine or 300 IU of A/Eng/72 vaccine produced serum HI antibody to the vaccine virus (Table 1). Serum HI antibody titres to the immunizing virus ranged from 1/480 to 1/1920 for A/Eng/72-immunized ferrets and 1/240 to 1/5120 for ferrets given A/HK/68 vaccine. In both groups of ferrets, HI titres were highest against the immunizing virus, while the titres of antibody cross-reacting with the heterotypic virus were 1·5- to 8-fold lower.

Ferrets immunized with 300 CCA of A/HK/68 vaccine or 300 i.u. of A/Eng/72 vaccine, both given with Freund's incomplete adjuvant, also produced high titres of serum HI antibody (Table 1). Antibody titres to the immunizing virus ranged from 1/320 to 1/4800 for ferrets inoculated with A/Eng/72 vaccine, and from 1/120 to 1/3840 for animals given A/HK/68 vaccine. A varying degree of cross-reactivity was found between antibody to the immunizing virus and the heterotypic virus; in some cases the antibody titres to both viruses were similar.

Table 1. *Response of ferrets to infection or immunization with A/Eng/42/72 or A/HK/1/68 viruses or virus vaccines*

Ferret no.	Treatment	Serum HI antibody response to* A/Eng/72	A/HK/68
375	A/Eng/42/72 infection	< 10–1920	< 10–240
376		< 10–1600	< 10–2400
377		< 10–3840	< 10–960
378		< 10–1280	< 10–320
379	A/HK/1/68 infection	10–320	< 10–240
380		15–600	10–800
381		< 10–640	< 10–960
382		< 10–1200	< 10–2400
407	A/PR/8/34 infection	15–1600	20–1280
408	A/Eng/72 vaccine	< 10–480	< 10–320
409		< 10–960	< 10–120
410		15–1920	< 10–1280
411	A/PR/8/34 infection	< 10–30	15–240
412	A/HK/68 vaccine	15–960	30– > 5120
413		< 10–480	< 10–1920
414		< 10–120	10–320
401	A/Eng/72 vaccine with	< 10–3200	< 10–1920
402	Freund's incomplete	< 10–4800	10–6400
403	adjuvant	< 10–320	< 10–40
		15–1280	< 10–320
399	A/HK/68 vaccine with	< 10–240	10–3840
400	Freund's incomplete	< 10–240	< 10–480
405	adjuvant	< 10–240	< 10–120
406		< 10–960	< 10–1280

* Antibody titre before infection/immunization–antibody titre 8–9 weeks after infection, or 5 weeks after immunization.

Response of ferrets to challenge infection with influenza virus A/Mill Hill/1/72

Normal ferrets

Four normal ferrets were infected by intranasal inoculation of approximately $4 \times 10^{7.00}$ EID50 of influenza virus A/Mill Hill/1/72. High titres of virus were recovered from nasal washings collected on the 2nd day after infection from all four ferrets; the titres of virus in these specimens ranged from $10^{4.50}$–$10^{6.16}$ EID50/ml. (geometric mean titre (gmt) = $10^{5.24}$ EID50/ml.). The animals produced high titres of serum HI antibody to A/Eng/72 virus, with lower titres of antibody to A/HK/68 virus (Table 2). Following infection, the mean concentration of protein in nasal washings from the four ferrets increased more than threefold with peak levels present on day 7 (Fig. 1). HI antibody was also detected in nasal washings from all four ferrets, with peak antibody titres to A/Eng/72 of 1/10 to 1/160 occurring on day 11; HI antibody titres to A/HK/68 virus parallelled those to A/Eng/72, but were lower (Fig. 1).

Table 2. *Response of ferrets to A/Mill Hill/1/72 infection after previous infection with influenza A virus*

Ferret no.	First infection	Virus isolation (log_{10} EID 50/ml.)		Response to challenge infection with A/Mill Hill/1/72: Change in serum HI titre* A/Eng/72	Change in serum HI titre* A/HK/68	Change in nasal HI titre† A/Eng/72	Change in nasal HI titre† A/HK/68
383	Nil	5·16	(5·24)	< 10–5120	< 10–960	< 5–40	< 5–10
396		6·16		< 10–3840	< 10–160	< 5–80	< 5–15
397		5·16		< 10–5120	< 10–120	< 5–160	< 5–60
398		4·50		15–5120	15–1920	< 5–10	—‡
375	A/Eng/72	< 0·7	(1·28)	1920–5120	240–480	< 5–7·5	< 5–7·5
376		3·16		1600–12800	2400–12800	< 5–30	< 5–20
377		< 0·7		3840–1920	960–120	—	—
379	A/HK/68	4·5	(3·28)	320–> 5120	240–> 5120	—	< 5–7·5
380		4·16		600–6400	800–12800	—	< 5–5
381		3·83		640–> 5120	960–5120	—	< 5–10
382		< 0·7		1200–9600	2400–12800	< 5–15	< 5–20

* Antibody titre before challenge infection–antibody titre 15 days after infection.
† Antibody titre before challenge infection–peak antibody titre after infection.
‡ — = < 5–< 5.

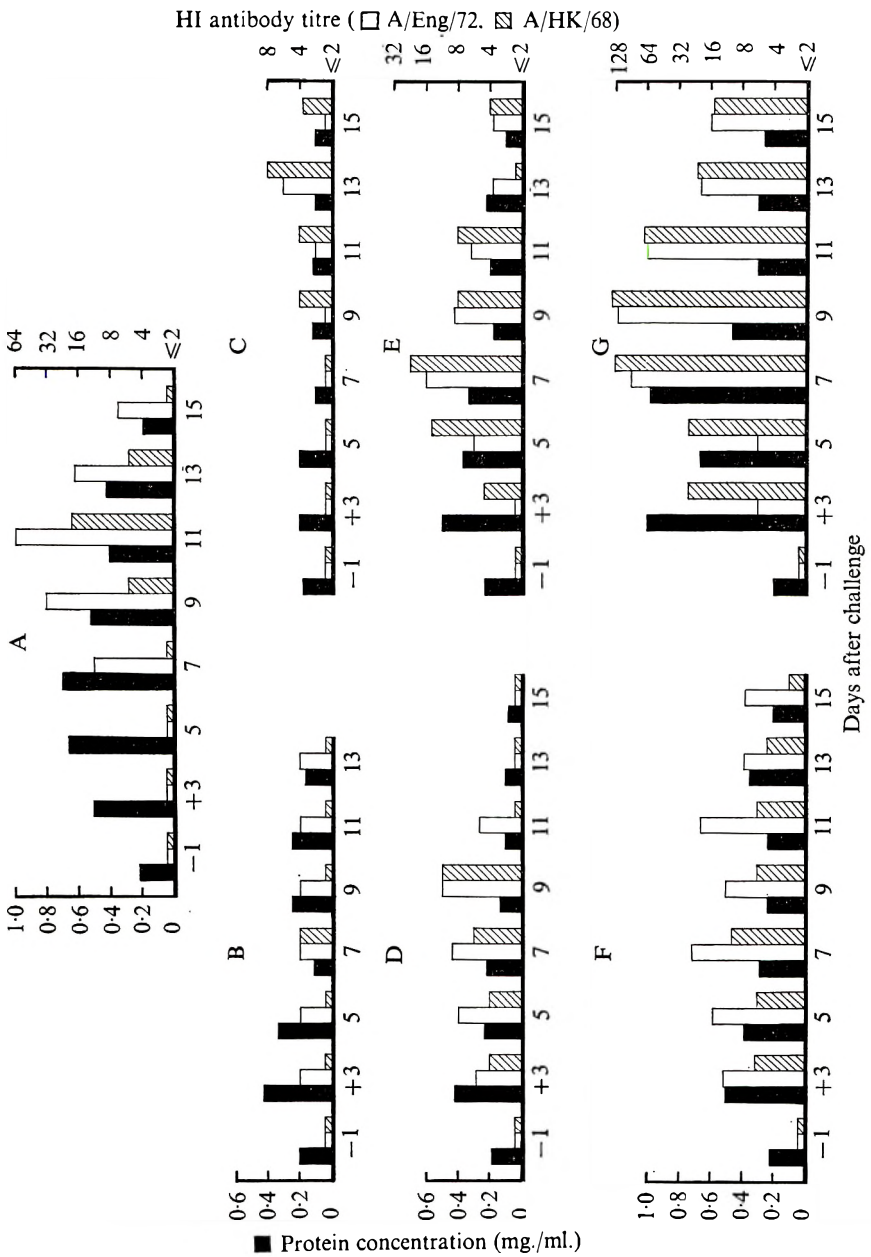

Fig. 1. Nasal response of ferrets to challenge infection with influenza virus A/Mill Hill/1/72. A, Normal ferrets; B, ferrets previously infected with A/Eng/42/72; C, ferrets previously infected with A/HK/68; D, ferrets previously infected with A/PR/8/34 then immunized with A/Eng/72 vaccine; E, ferrets previously infected with A/PR/8/34 then immunized with A/HK/68 vaccine; F, ferrets immunized with A/Eng/72 vaccine in Freund's incomplete adjuvant; G, ferrets immunized with A/HK/68 vaccine in Freund's incomplete adjuvant.

Ferrets previously infected with A/Eng/72 influenza virus

Three ferrets which had been infected with A/Eng/72 virus 10 weeks previously were challenged by intranasal inoculation of approximately $4 \times 10^{7.0}$ EID50 of influenza virus A/Mill Hill/1/72. After infection, virus was re-isolated from nasal washings taken on the 2nd day from only one of the three ferrets (Table 2). A significant increase in serum HI titre occurred in one of the animals, and two of the ferrets produced detectable levels of HI antibody in nasal washings collected after challenge infection. The mean concentration of protein in nasal washings increased approximately twofold after infection, but soon fell to pre-infection levels (Fig. 1).

Ferrets previously infected with A/HK/68 influenza virus

Four ferrets which had been infected with A/HK/68 virus 8 weeks previously were challenged by infection with A/Mill Hill/72 virus. Virus was recovered from nasal washings from three of the animals, and a four-fold or greater increase in serum HI titre was found for all four animals (Table 2). Similar increases in serum HI antibody were measured to both A/Eng/72 and A/HK/68 viruses. HI antibody to A/Eng/72 was detected in the nasal washings collected after challenge infection from only one of the four ferrets in this group; virus was not recovered from nasal washings from this ferret. In contrast, all the ferrets in this group produced detectable levels of HI antibody to A/HK/68 in nasal washings, with the maximum, mean HI titre occurring on day 13. No significant increases in the concentration of nasal wash protein were measured in specimens from these animals (Fig. 1).

Ferrets immunized with either A/Eng/72 or A/HK/68 virus vaccines

Regardless of the method of immunization, all the ferrets which had been given either A/HK/68 or A/Eng/72 vaccine were subsequently infected by the A/Mill Hill/72 challenge virus (Table 3). Thus, virus was recovered from nasal washings collected from all the ferrets, although the geometric mean titres of virus recovered from each group was measurably lower than from normal ferrets infected with the same virus. The differences in the titres of virus recovery for the different groups of immunized ferrets were not significant. All the animals showed an increase in serum HI antibody titre to A/Eng/72 virus following infection; however, for some of the animals given A/Eng/72 vaccine, either with adjuvant or after A/PR/8/34 infection, the rise in antibody titre was less than fourfold (Table 3).

For ferrets infected with A/PR/8/34 virus before immunization, the peak concentrations of protein and titres of HI antibody present in nasal washings collected after challenge were lower than those found after challenge of normal ferrets. In addition the peak concentrations of protein and HI antibody titres occurred 2–4 days earlier than was seen in normal ferrets after challenge infection (Fig. 1). The group of ferrets immunized with A/Eng/72 vaccine in Freund's incomplete adjuvant had concentrations of nasal wash protein similar to those found in ferrets given the same vaccine dose in saline after A/PR/8/34 infection; however, significantly higher titres of HI antibody to A/Eng/72 were produced in nasal washings from the former group (Fig. 1). In both groups of animals, the

Table 3. *Response of ferrets to A/Mill Hill/1/72 infection following immunization*

Ferret no.	Immunization	Virus isolation (log_{10} EID 50/ml.)		Response to challenge infection with A/Mill Hill/1/72: Change in serum HI titre* A/Eng/72	Change in serum HI titre* A/HK/68	Change in nasal HI titre† A/Eng/72	Change in nasal HI titre† A/HK/68
407	A/PR/8 infection	4·83	(4·33)	1600–4800	1280–6400	—‡	—
408	A/Eng/42/72 vaccine	4·50		580–> 5120	320–960	< 5–7·5	—
409		3·50		960–3840	120–240	< 5–30	< 5–10
410		4·50		1920–> 5120	1280–> 5120	< 5–40	< 5–30
411	A/PR/8 infection	4·16	(4·41)	30–> 5120	240–> 5120	—	—
412	A/HK/68 vaccine	3·50		960–> 5120	> 5120–> 5120	< 5–60	< 5–80
413		4·83		480–> 5120	1920–> 5120	—	—
414		5·16		120–> 5120	320–> 5120	< 5–5	< 5–20
401	A/Eng/42/72 vaccine with	4·83	(4·08)	3200–4800	1220–3840	< 5–30	< 5–15
402	Freund's incomplete	4·16		4800–38400	6400–19200	< 5–30	< 5–10
403	adjuvant	3·83		320–5120	40 320	< 5–30	< 5–5
404		3·50		1280–3840	320–640	< 5–40	< 5–10
399	A/HK/68 vaccine with	4·50	(3·92)	240–> 5120	480–> 5120	< 5–120	< 5–160
400	Freund's incomplete	4·16		240–5120	480–> 5120	< 5–60	< 5–120
405	adjuvant	4·50		240–> 5120	120–> 5120	< 5–320	< 5–320
406		2·5		960–> 5120	1280–> 5120	< 5–30	< 5–30

* Antibody titre before challenge infection–antibody titre 15 days after infection.
† Antibody titre before challenge infection–peak antibody titre after infection.
‡ —, < 5–< 5.

HI antibody titres to A/Eng/72 virus in nasal washings were higher than the titres to A/HK/68 virus.

Ferrets previously immunized with A/HK/68 vaccine in adjuvant had a more pronounced nasal response after challenge infection than any of the other groups of ferrets. Thus a mean peak concentration of protein of 1·0 mg./ml. was found in nasal washings collected on day 3, and high titres of HI antibody (1/30–1/320) were found in nasal washings, with peak titres on days 7 and 9 (Fig. 1). Antibody titres were similar to both A/Eng/72 and A/HK/68 viruses. All of the ferrets in this group showed a fourfold or greater rise in A/Eng/72 serum HI titre after challenge infection (Table 3).

DISCUSSION

Immunity to A/Eng/72 virus was produced in ferrets by infection with live virus or by immunization with inactivated virus vaccines. The results confirm earlier observations that live infection gives a more complete and broader immunity than does immunization, although similar serum HI antibody titres are produced by each procedure (Potter *et al.* 1972*b*). Thus, ferrets infected with influenza virus A/Eng/72 8 weeks before challenge with the similar virus A/Mill Hill/72 were found to be almost completely immune to the challenge infection. Virus was re-isolated in low titre and a significant rise in serum HI antibody titre to A/Eng/72 virus was obtained in only one of three ferrets after infection. The successful re-infection of this animal may be due to the large amount of virus (10^7 EID50) used for the challenge; previous attempts to re-infect ferrets with 10^6 EID50 of influenza virus A/HK/68 5–10 weeks after primary infection with the same virus were not successful (Potter *et al.* 1972*a*). Alternatively the A/Mill Hill/72 virus strain may be more virulent for ferrets than the A/Eng/72 virus. Ferrets were also infected with A/HK/68 virus before challenge with A/Mill Hill/72 virus. The first infection produced relatively high titres of cross-reacting serum HI antibody to the challenge virus, but lower titres than seen after A/Eng/72 infection; the A/HK/68-convalescent ferrets were partially immune to A/Mill Hill/72, but the immunity was not as great as found in A/Eng/72-convalescent animals.

Serum HI antibody to A/Eng/72 virus was also produced in ferrets by immunization. Previous reports have shown that ferrets only respond to conventional doses of influenza vaccines if the vaccine is given with adjuvant (Potter, McLaren & Shore, 1973), or if the animal has previously been infected with a heterotypic influenza A virus (McLaren & Potter, 1974). After immunization by either of these methods with A/Eng/72 or A/HK/68 vaccine, the ferrets produced serum HI antibody titres similar to those found in ferrets previously infected with live virus; however, these ferrets did not have the same degree of immunity to A/Mill Hill/72 challenge infection as that produced after infection. Thus, although ferrets previously infected with A/HK/68 virus had lower cross-reacting serum antibody titres to A/Eng/72 than did ferrets immunized with A/Eng/72 vaccine in adjuvant, they were significantly more resistant to A/Eng/72 challenge infection than were the immunized animals. It was also observed that the degree of

immunity to challenge infection was the same for ferrets whether they were given A/Eng/72 or A/HK/68 vaccine. Previous studies in mice have also shown that the immunity resulting from live infection is broader in activity than that produced by vaccines, and can give partial protection against heterotypic influenza viruses (Oakley & Warrack, 1940; Schulman & Kilbourne, 1965; Werner, 1966; Schulman, 1967) but the reason for the greater immunity is not clear. In ferrets it may be due to the wide range of activity reported for their nasal antibody (Haff & Pinto, 1973) which is produced after infection but not after immunization. Alternatively infection may be more effective at stimulating cellular immunity than immunization with inactivated vaccines.

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An analysis of the susceptibilities of several populations of *Rattus norvegicus* to warfarin

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SUMMARY

An analysis was made of the dose-response of several populations of *Rattus norvegicus* fed upon baits containing 0·005 % warfarin for various numbers of days. Warfarin-susceptible populations fell within a narrow range, with LFP50s and LFP98s (lethal feeding periods in days to obtain 50 % and 98 % mortalities respectively) of up to 3·0 and 5·0 days respectively. The probability of an individual rat from these populations surviving a six-day feeding period was estimated at 0·003 or less. Populations with responses falling beyond these limits were regarded as warfarin-resistant.

Six of nine populations of *R. norvegicus*, from England, Germany and the United States, were determined to be warfarin-susceptible within the narrow limits given above. In all six cases, no animals survived the six-day WHO feeding test for anticoagulant susceptibility. In three populations from the United States, where rats survived six days feeding, their population responses clearly fell outside the measures given above. It is suggested, tentatively, that anticoagulant-resistant Norway rat populations be defined as those whose LFP50 and LFP98 exceeds 3·0 and 5·0 days respectively, and in which the probability of an individual animal surviving a six-day feeding upon 0·005 % warfarin is 0·01 or more.

INTRODUCTION

The purpose of the anticoagulant-resistance feeding test as originally proposed by Drummond (1966) and later modified and adopted by the World Health Organization (1970) is to measure the susceptibility of rodent populations to a given anticoagulant rodenticide. The proposed concentrations of anticoagulants and number of days of feeding were left open to the discretion of investigators until additional experience dictated the appropriate concentrations or time periods.

In a subsequent paper, Drummond & Wilson (1968) suggested that warfarin at 0·005 % concentration (by weight) in a suitable bait fed to *Rattus norvegicus* for six days provided a suitable screening test for detecting resistance in that species. Bentley (1969) subsequently defined resistant Norway rats in the United Kingdom as those that survived a standard feeding period of six days on 0·005 % warfarin in the laboratory. Other investigators, including ourselves (Brooks & Bowerman, 1973), have used this test as a basis for detection of anticoagulant-resistant rats in the United States. More recently, the six-day feeding test at

0·005 % warfarin has been used as a screening test to detect developing anticoagulant resistance in Norway rats in cities in the United States (Jackson, Brooks, Bowerman & Kaukeinen, 1973).

The original basis for using a six-day feeding at 0·005 % warfarin concentration rests upon the evidence provided by Drummond & Wilson (1968) of one population of susceptible Norway rats in England. Further confirmation was provided by ourselves (Brooks & Bowerman, 1973) from the testing and analysis of two warfarin-naïve Norway rat populations in New York State. In this present paper we re-examine the published data on the susceptibility of *Rattus norvegicus* to warfarin and also look at new evidence derived by ourselves on additional Norway rat populations. Our purpose will be to present an analysis of base-line warfarin susceptibility in *Rattus norvegicus* and to derive statistical criteria by which both normal warfarin-susceptible rat populations may be defined and those by which resistant populations may be characterized.

MATERIALS AND METHODS

Norway rats, both wild-caught and a domestic laboratory strain (Long-Evans), were individually caged after first being weighed and sexed. Sick, pregnant and immature animals were set aside. A basal diet of laboratory meal was provided for each animal and water was available *ad lib*. Animals were acclimated to cage conditions for a minimum of three weeks before testing.

The test procedure employed a pre-test baiting with either ground rolled oats or Purina laboratory chow. Rolled oats were used in our earlier testing and Purina chow has been used routinely as a standard test diet since April 1972. After feeding was stabilized, the amount eaten daily was measured for two days preceding the trial. Then, for periods ranging from one to twelve days, groups of rats were allowed unrestricted feeding upon a bait containing 0·005 % warfarin, by weight. Food consumption was measured daily, accounting also for any spillage caught on papers below each cage. The warfarin was supplied by the Wisconsin Alumni Research Foundation as a technical powder and was mixed into a master concentrate with corn starch or finely ground Purina lab chow. Animals were observed daily during the trial and for a ten-day period after the last warfarin feeding for symptoms of anticoagulant poisoning (bleeding, pilo-erection, sluggishness, bleached extremities) and for mortality. Dead animals were autopsied to verify anticoagulant effects. After the poisoning trial, animals were returned to the basal diet of laboratory meal. Animal weights were determined immediately before the warfarin baiting and a terminal weight was obtained. In some cases, animals weighed less than 150 g., but having been caged at least 60 days, were used in the trials.

All mortality data were evaluated using the dose-response analysis as proposed by Litchfield & Wilcoxon (1949). Data were first evaluated for males and females separately, and if no significant differences were found in slope function ratios and potency ratios, their combined data were used. Significant differences occurred only between males and females from Cambridge, New York and results for them are presented separately.

Table 1. *Mortality to Norway rats from several populations after unrestricted feeding on baits containing* 0·005 % *warfarin for various numbers of days*

Population	Days feeding	Mortality					
		Males		Females		Both sexes	
		No.	Percent	No.	Percent	No.	Percent
Refuse destructor, English Midlands (Drummond & Wilson, 1968)	1	0/2	0·0	5/10	50·0	5/12	41·7
	2	3/7	42·8	16/22	72·7	19/29	65·5
	3	16/16	100·0	14/14	100·0	30/30	100·0
	4	7/7	100·0	4/4	100·0	11/11	100·0
	6	3/3	100·0	4/4	100·0	7/7	100·0
Refuse disposal site, Merrick, N.Y. (Brooks & Bowerman, 1973)	1	3/12	33·3	1/11	9·1	5/23	21·7
	2	19/22	86·3	5/10	50·0	24/32	75·0
	3	10/10	100·0	10/10	100·0	20/20	100·0
Chicken farm, Sharon Springs, N.Y.	1	1/8	12·5	2/8	25·0	3/16	18·7
	2	9/10	90·0	5/10	50·0	14/20	70·0
	4	8/8	100·0	9/10	90·0	17/18	94·4
	6	11/11	100·0	11/11	100·0	22/22	100·0
Refuse tip, Borkum, Germany (Telle, 1971)	2	12/29	41·4	20/32	62·5	32/61	52·4
	3	10/11	90·9	8/8	100·0	18/19	94·7
	4	15/15	100·0	16/16	100·0	31/31	100·0
	5	40/40	100·0	18/18	100·0	58/58	100·0
	6	4/4	100·0	6/6	100·0	10/10	100·0
Refuse disposal site, Berlin, N.Y. (Brooks & Bowerman, 1973)	2	1/10	10·0	1/7	14·3	2/17	11·8
	3	9/10	90·0	7/8	87·5	16/18	88·8
	4	10/10	100·0	10/14	71·4	20/24	83·3
	5	5/5	100·0	9/9	100·0	14/14	100·0
Long-Evans laboratory rats	1	—	—	0/10	0·0	0/10	0·0
	2	1/10	10·0	4/10	40·0	5/20	25·0
	3	8/10	80·0	8/10	80·0	16/20	80·0
	4	10/12	83·3	10/10	100·0	20/22	90·9
	5	9/10	90·0	—	—	—	—
	6	12/12	100·0	2/2	100·0	14/14	100·0
Refuse disposal site, Pittstown, N.Y. (Brooks & Bowerman, 1973)	2	3/10	30·0	3/12	25·0	6/22	27·2
	3	9/15	60·0	5/12	41·7	14/27	51·8
	4	9/10	90·0	9/11	81·8	18/21	85·7
	5	11/11	100·0	9/10	90·0	20/21	95·2
	6	9/9	100·0	10/11	90·9	19/20	95·0
	7	—	—	14/14	100·0	14/14	100·0
Feed mill, Albany, N.Y.	2	0/6	0·0	0/8	0·0	0/14	0·0
	4	4/8	50·0	2/8	25·0	6/16	37·5
	5	7/8	87·5	4/8	50·0	11/16	68·7
	6	15/15	100·0	24/25	96·0	39/40	97·5
Turkey farm, Cambridge, N.Y. (Brooks & Bowerman, 1973)	4	1/8	12·5	1/8	12·5	2/16	12·5
	6	6/8	75·0	5/8	62·5	11/16	68·7
	8	8/8	100·0	6/8	75·0	14/16	87·5
	10	12/12	100·0	10/12	83·3	22/24	91·6
	12	—	—	9/10	90·0	—	—

Table 2. *Base-line susceptibilities of several populations of* Rattus norvegicus *to* 0·005 % *warfarin* (95 % *confidence limits in days given for each lethal feeding period*)

The probability of a rat from each population surviving a six-day feeding upon 0·005 % warfarin is estimated using a log-normal distribution fit, where the mean is estimated as the log LFP 50 and the standard deviation is estimated as the log-slope function(S).

Population	Number of rats	Slope function (S)	LFP 50 (days)	LFP 98 (days)	Probability of survival
Merrick, New York	75	1·42	1·44 (1·26–1·64)	3·00 (2·29–3·93)	0·0005
Borkum, Germany	179	1·29	1·97 (1·79–2·17)	3·36 (2·77–4·06)	0·0005
English Midlands	89	1·72	1·35 (1·05–1·73)	3·80 (2·14–6·72)	0·003
Sharon Springs, New York	76	1·64	1·54 (1·22–1·94)	4·25 (2·74–6·59)	0·003
Long Evans rats	96	1·37	2·46 (2·14–2·83)	4·75 (3·89–5·79)	0·002
Berlin, New York	73	1·34	2·58 (2·28–2·92)	4·82 (3·79–6·13)	0·002
Pittstown, New York	125	1·48	2·77 (2·43–3·16)	6·20 (3·95–9·73)	0·024
Albany, New York	86	1·26	4·35 (3·91–4·91)	7·00 (5·69–8·61)	0·082
Cambridge, New York (males)	36	1·24	5·15 (4·15–6·38)	8·05 (5·88–11·03)	0·238
Cambridge, New York (females)	46	1·80	5·40 (4·91–5·94)	17·7 (—)	0·428

In all cases, lines were fitted to the observed mortalities on log-probability paper until the best fit was obtained. Then the LFP 50s and LFP 98s (Lethal Feeding Periods, in days, to obtain 50 % and 98 % mortalities, respectively) were obtained and the 95 % confidence limits at these dosage levels were estimated (Litchfield & Wilcoxon, 1949). We are following the terminology as suggested by the British workers (Rowe & Redfern, 1964; Drummond, personal communication) of Lethal Feeding Period, rather than Effective Dose, as being a better description of the nature of the measurement. The LFP 50 is also known as the mean Lethal Feeding Period. The slope functions, which are 'the fold change in the line required to produce a unit standard deviation change in response along the line' (Litchfield & Wilcoxon, 1949) are given also as part of the descriptive parameters.

RESULTS AND DISCUSSION

Data are presented on nine Norway rat populations (Table 1). Six populations were examined from published literature (Drummond & Wilson, 1968; Telle, 1971 and Brooks & Bowerman, 1973) and three are populations recently tested by ourselves.

Populations are ranked by increasing order according to their LFP 98s in Table 2. The first six populations could all be described as warfarin-susceptible, since in all cases 100 % mortality was achieved in six days' feeding or less. These six warfarin-susceptible populations are drawn from widely separated geographic areas and represent rats both with and without past exposure to anticoagulants. Telle (1971) describes the rats from Borkum as coming from an indigenous

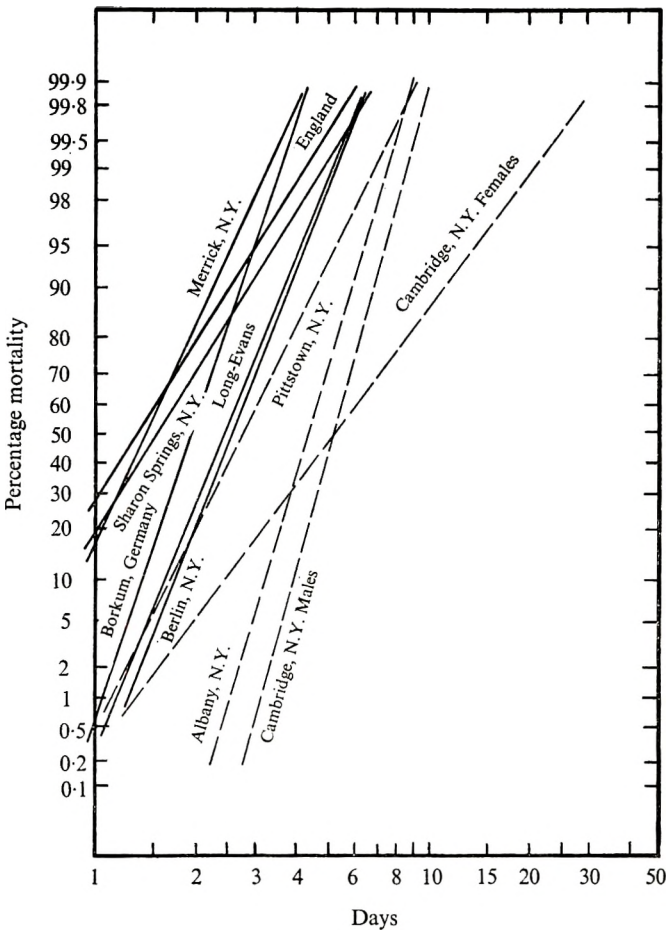

Fig. 1. Dose–response lines of several populations of *Rattus norvegicus* to 0·005 % warfarin. Solid lines are considered warfarin-susceptible; dashed lines are defined as warfarin-resistant.

population on a refuse tip; survivors were caught after routine control campaigns. The rats from Sharon Springs, New York, were trapped on a chicken farm and had a history of 10 to 15 years' past exposure to anticoagulants on an irregular basis.

These six populations indicate that the base-line susceptibility of *Rattus norvegicus* to 0·005 % warfarin is rather narrowly defined. Their LFP50s are characterized as between 1·35 to 2·58 days (with an upper 95 % confidence limit of 2·92 days). Their LFP98s, which are of most concern to us, fall between 3·0 and 4·82 days (with an upper 95 % confidence limit of 6·72 days). In Fig. 1, these fitted dose-response lines are shown graphically.

An examination of the three populations where one or more rats survived a six-day feeding period on 0·005 % warfarin indicates that they fall outside the normal narrowly circumscribed limits of the susceptible groups. Pittstown, a refuse-disposal-site rat population with a history of moderate anticoagulant pressure in the recent past, exhibits a dose-response line falling within the 95 %

confidence limits of susceptible rats, but the LFP98 is 6·20 days, a value significantly increased from the susceptible groups. Pittstown, however, is considered as an example of incipient resistance; a site to be monitored again in a year or so.

The Albany population was trapped from a feed mill in the port of Albany, New York. The population here had been periodically poisoned with anticoagulants and acute rodenticides by a commercial pest-control operator for a number of years. Here the population response to warfarin has clearly shifted toward resistance. The LFP50 has increased to 4·38 days and the LFP98 to 7·0 days. The dose-response line falls outside the 95% confidence limits for susceptible rats at these dosage levels.

Finally, the resistance site at Cambridge, New York, a turkey farm, is an example of a warfarin-resistant population. Approximately 30% of the rats here survived a six-day feeding period. The response of the sexes was such that they required separate analysis. Males had an LFP50 of 5·15 days, a value reasonably close to that of the females, 5·40 days. However, their slope functions differed significantly. Their respective LFP98s were 8·05 days for males and 17·7 days for females. No confidence limits were estimated for Cambridge females because they were meaningless.

In Table 2 is also given the probability of a rat from each of the several populations surviving a six-day feeding period using the log-normal distribution fit, where the means are estimated as the log-LFP50 and the standard deviations are estimated as log-slope function. In this example, the probabilities for the first six populations do not exceed 0·003. Thus the chances of 'normal' rats surviving a six-day feeding test are extremely remote. In contrast, the probabilities of survival increase from 0·02 in Pittstown up to 0·43 in Cambridge females.

Based upon these evaluations of the responses of nine Norway rat populations to 0·005% warfarin, we would suggest that warfarin-susceptible Norway rat populations tentatively be defined as those whose dose-responses do not exceed LFP50s of 3·0 days and LFP98s of 5·0 days and whose individual member's probability of survival of a six-day feeding period is no more than 0·01. It is suggested that population responses falling beyond these measures should be regarded as warfarin-resistant. Furthermore, the validity of the six-day feeding test of 0·005% warfarin in oatmeal or Purina lab chow is clearly established and the survival of even one animal, as Drummond states (1966), should be regarded as an alert calling for further investigation. It should be pointed out that the data reviewed and presented here are applicable only to populations of *Rattus norvegicus*. Comparable dose-response data need to be developed for other species of pest rodents which are frequently and repeatedly poisoned with anticoagulants.

We wish to acknowledge the advice and assistance of Mr Philip Quickenton, Biostatistician, New York State Department of Health, in computing the probabilities of survival of the various rat populations. We wish to thank D. C. Drummond, W. M. Jackson and D. E. Kaukeinen for kindly reviewing the manuscript and providing us with their critical remarks. We especially and gratefully acknowledge the technical assistance of our staff members in the laboratory

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Clearance of antibiotics from the intestines after termination of antibiotic decontamination

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SUMMARY

The clearance of neomycin and kanamycin from the intestines after stopping oral supply has been determined in mice. Both antibiotics, although given in different doses, were excreted in essentially the same way; the clearance being a little faster than logarithmically in both cases. The importance of this observation with regard to isolation and the moment of reconventionalization is discussed.

INTRODUCTION

The influence of the intestinal microflora on gastro-intestinal motility in mice has been previously investigated by Abrams & Bishop (1966, 1967). Using conventional and germ-free mice they demonstrated that the conventional microflora contributes to the control of the population of *Salmonella typhimurium* in the small intestine primarily by stimulating peristaltic emptying. The rate of propulsion of gastrointestinal contents compared in germ-free and conventional mice, using a non-absorbable radioactive substance yttrium 91, was found to be much higher in the conventional mice than in the germ-free animals. It took 24 hr. in the conventional mice before 100 % of the radioactive label was recovered in the faeces of germ-free animals.

In a previous communication (van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees, 1971*b*), we have mentioned the possibility that also in antibiotic decontaminated animals a reduction of the enteric motility may explain the considerable decrease of the Colonization Resistance (C.R.) of the digestive tract which occurs during antibiotic decontamination.

In this paper we report attempts to measure the clearance of antibiotics from the intestines in decontaminated mice. This was accomplished by determining the concentration of non-absorbable antibiotics in the faeces daily after stopping oral treatment. The transit time in the upper part of the digestive tract was determined with a non-toxic non-absorbable dye, vermilion red.

MATERIALS AND METHODS

Animals

Conventional and germ-free ND2 female mice aged 12 weeks were used. Body weight varied between 32 and 40 g. During treatment, the animals were maintained in autoclaved cages with 4–5 animals to a cage inside a laminar cross-flow bench (van der Waaij & Andreas, 1971*a*). The chamber of the bench was peracetic acid sterilized and food, water and bedding were autoclaved. The animals were handled with sterile gloves. The germ-free mice used in one experiment were maintained similarly.

Antibiotics

On the basis of the outcome of an antibiotic sensitivity test of the faecal flora of the animals (van der Waaij, de Vries & Lekkerkerk, 1970), two combinations were used. In one experiment, performed with 10 animals, the combination of kanamycin and bacitracin was used while, in another experiment, a similar group was treated with neomycin and bacitracin. In the experiments in which the antibiotic concentration in the faeces was determined, these antibiotics were given for 2 weeks in the drinking water. Kanamycin was given at a concentration of 1 mg./ml., in combination with 1 mg./ml. bacitracin; neomycin was given at a concentration of 5 mg./ml. in combination with 5 mg./ml. bacitracin. In each instance 100 μg./ml. of pimaricin was added to suppress growth of yeasts and fungi.

The mice used in the vermilion red dye test were treated with neomycin and bacitracin both 2·5 mg./ml. and pimaricin 100 μg./ml. in the drinking water.

Antibiotic concentration assay

To determine the concentration of kanamycin and neomycin in the faeces of the mice, the microcup dilution method of Goss & Cimijotti (1968) was used after it was standardized and reference curves were made for a sensitive strain of *Escherichia coli*.

After stopping the oral supply of antibiotics, fresh faeces were sampled daily for 5 days after the last day of treatment. The faeces were suspended 1/10 in tryptose phosphate (T.P.) broth (Difco). After low-speed centrifugation, the supernatant of these suspensions was then assayed.

Vermilion red test

The propulsive activity of the small intestines was investigated by giving the animals 0·2 ml. of 2 % vermilion red solution by stomach tube. At 1, 5, 10, 20, 40, 60 and 120 min intervals after administration the animals were killed in groups of 10 by cervical dislocation. The intestines were removed immediately after death and put in formaldehyde to stop further propulsion of the dye. Then the distance along which the dye had moved was measured. This experiment was performed with conventional, germ-free and decontaminated mice. The decontaminated animals were treated in two groups of 70. One group had been decontaminated for 4 days, the other for 2 weeks.

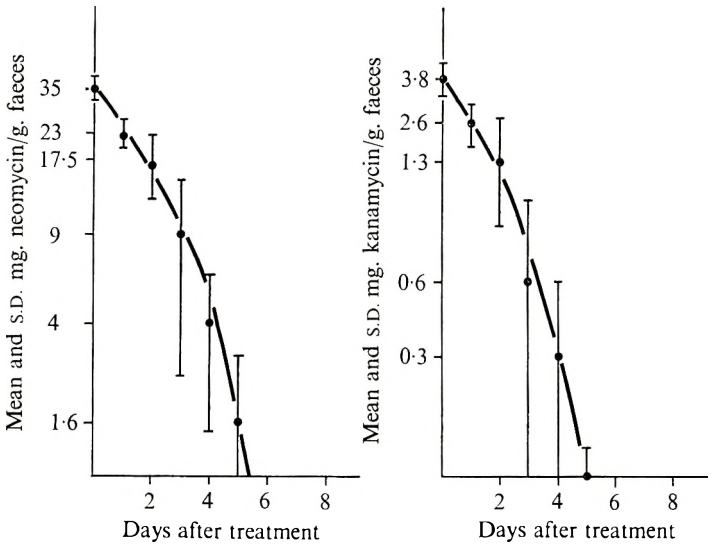

Fig. 1. Clearance of antibiotics from the intestines of mice expressed as the mean faecal concentration (and S.D.) at daily intervals after termination of treatment.

Caecal weight

The absolute and the relative weight of the caecum was determined at intervals of 2 days for 2 weeks after stopping oral antibiotic treatment, and thereafter weekly for 3 additional weeks. The animals were killed in groups of 8.

Bacteriological culturing

To investigate the effect of oral antibiotic treatment fresh faeces were sampled twice a week from each cage. The faeces from each cage were suspended in such a volume of Brain Heart Infusion broth (Difco) and Brewer's semi-solid thioglycollate that the antibiotic concentration existing in the faeces was diluted down to 1 μg./ml. or less. The suspensions were incubated at 37° C for at least 4 days before they were recorded as sterile. Also after antibiotic treatment was stopped mice were killed on alternate days in groups of 8 for aerobic culturing of intestinal contents.

RESULTS

The results of this study indicate that the clearance time of non-absorbable antibiotics from the gastro-intestinal tract is several days in decontaminated mice. In most of the animals treated with both the high dose (neomycin) and the low dose (kanamycin) it was more than 5 days before the antibiotics had disappeared from the faeces of all animals (Fig. 1).

The peristaltic activity of the small intestine was also much stronger in the conventional control mice than in those which were treated with antibiotics. The group of mice which were maintained decontaminated for 2 weeks (with neomycin and bacitracin) had a greatly decreased intestinal peristaltic activity. Peristaltic

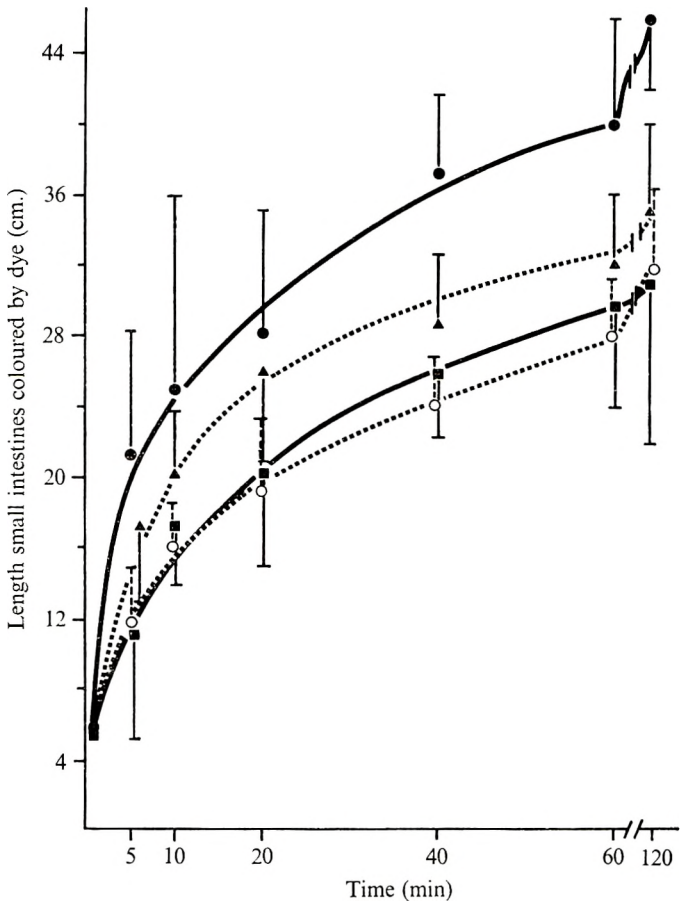

Fig. 2. Peristaltic activity of the small intestines expressed as the average distance (and S.D.) along which a dye was transported and the time. ●, Conventional mice; ■, germ-free mice; ▲, 4 days decontaminated mice; ○, 14 days decontaminated mice.

activity decreased already in the first week of treatment and was of the 'germ-free type' after 2 weeks (Fig. 2).

The results of cultures showed that decontamination had been successful in all animals in this study. Bacterial growth in the colon recurred only as late as 8 days after stopping treatment. However, the intestinal flora was still far from complete by that time, and the caecum was still enlarged. Reduction of the caecum started only 2 weeks after stopping treatment. The average caecal weight was back in the conventional range 3 weeks after antibiotic supply was stopped. The small intestines showed only occasionally positive cultures in animals killed in the first week after treatment. The percentage of positive cultures increased in the second week to become 100 % in the third.

DISCUSSION

The present study indicates that the propulsion of contents in both the small intestines and the colon is much slower in mice that have been decontaminated for 2 weeks than in conventional animals. A similar conclusion about the role of the microflora in intestinal peristalsis was made by Abrams & Bishop (1967) on the basis of a study in germ-free and conventional mice. These authors applied a radioactive labelled non-absorbable substance httrium and determined the disappearance of the labelled material in the various parts of the gastro-intestinal tract. This means that the resultant of peristaltic and antiperistaltic activity was measured. Antiperistalsis may have mixed the remaining labelled material with later ingested food substances and in that way prolonged the excretion period. In our experiments in which the disappearance of aminoglycoside antibiotics from the colon contents was investigated, a prolonged excretion was seen, similar to that found by Abrams & Bishop in germ-free mice. Both curves derived from the results of our experiments have a similar shape. The excretion of antibiotic substance apparently does not occur logarithmically. The mean antibiotic concentrations in the faeces during the first days after the termination of treatment were a little higher, and those of days 4 and 5 were lower, than would be expected of the excretion had occurred logarithmically (Fig. 1). This has the important practical implication, that the clearance of big antibiotic residues from the intestines is relatively more rapid than that of small amounts of antibiotics.

The long-lasting increased volume of the caecum after termination of oral antibiotic treatment under isolation conditions could be explained by the fact that the anaerobic species which are responsible for the small conventional caecum in mice (van der Waaij *et al.* 1971*b*) only gradually found favourable circumstances for growth in the intestines in the second week after treatment. Since the caecum is the site where the contents from the small intestines are mixed with the caecal contents, the size of the caecum will significantly influence the speed of excretion of residual antibiotic substances in the caecum. Because the caecal size remained constant in the first week after termination of treatment, reduction of caecum size cannot explain why the antibiotic excretion was found to occur faster than exponentially after day 3. It seems unlikely that a repair of peristaltic activity in the upper part of the intestinal tract in the first week after treatment has enhanced the clearance later in the week, because the differences in transit time of the small intestines between conventional and decontaminated mice were small although significant. Also the concentration of the (aminoglycoside) antibiotics in the caecum and colon cannot have played a significant role in the clearance pattern, because both the high dose (neomycin) as well as the low dose (kanamycin) derived curves have essentially the same shape. In conclusion, the mechanism responsible for the stronger than logarithmic clearance of antibiotics from the intestines after termination of treatment is still unknown and requires further investigation.

The observations described in this paper underline the necessity of continued isolation after the cessation of oral antibiotic treatment for decontamination. When in previously decontaminated mice an adequate (anaerobic) microflora is

implanted from day 5 after treatment on 3 consecutive days, a normal Colonization Resistance will return in the second week as was described previously (van der Waaij *et al.* 1971*b*). Termination of isolation before that time will result in an abnormal colonization of the digestive tract which is associated with spread into the regional lymphatic organs (van der Waaij *et al.* 1972). When this occurs in animals with a not yet completely restored defence apparatus and a more pathogenic microorganism is involved, infection will occur.

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Mycoplasmacidal activity of bovine milk for T-mycoplasmas

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SUMMARY

Normal bovine milk and whey was mycoplasmacidal for 6 of the 13 strains of bovine T-mycoplasmas examined. The *in vitro* assay used also demonstrated no killing of the human, canine and simian T-mycoplasma strains after 4 hr. incubation. However, there appeared to be some cow-to-cow variation in possession of this activity, and following *E. coli* endotoxin stimulation of the mammary gland the activity was considerably reduced.

Whey from three normal cows was fractionated on a Bio-Gel A 1·5 m. column and the mycoplasmacidal activity of the resulting five peaks assayed. Only the second peak, peak B, contained activity and was characterized as the only peak containing bovine IgA. The active component in whey, however, was found to be heat stable at 60° C. for 60 minutes and to pass through a dialysis membrane. This is inconsistent with it being immunoglobulin.

INTRODUCTION

Recently, certain T-mycoplasmas have been shown to cause experimental mastitis in cattle (Gourlay, Howard & Brownlie, 1972). These authors found variation both in the virulence of different T-mycoplasma strains and in the susceptibility of different cows (Howard, Gourlay & Brownlie, 1973). In an attempt to explain these variations, certain soluble factors in milk have been examined. The presence of soluble mycoplasmacidal factors has already been reported in serum from immune cattle (Priestley, 1952), in murine tissue extracts (Tully & Rask-Nielsen, 1967) and in rabbit and human neutrophil extracts (Dajani & Ayoub, 1969; Jones & Hirsch, 1971). Particularly pertinent to our own observations, to be reported here, was the finding that bull seminal fluid and serum could inhibit the growth of T-mycoplasmas (Taylor-Robinson, Thomas & Dawson, 1969).

In cow's milk, however, only bactericidal activity has so far been reported. Hesse (1894) was the first to report this effect and Hanssen, in 1924, called the responsible fraction 'lactenin'. Many observations have confirmed these findings with normal cow's milk, while Derbyshire (1964) demonstrated an even greater bactericidal activity in the milk of cows with a sterile inflammation of the mammary gland. This mastitis, induced by intramammary infusions of distilled water, was due to the pyrogens contained in distilled water (Shah & Morse, 1964).

The mycoplasmacidal activity of milk from the normal bovine mammary gland, from the gland after endotoxin stimulation, and after T-mycoplasma experimental infection was investigated in an attempt to explain the variable pathogenicity of T-mycoplasmas for the mammary gland.

METHODS

Cattle

The cattle were 3 to 6-year-old Friesian cross in the first 8–12 weeks of their second to fifth lactation. All animals studied were giving more than $2\frac{1}{2}$ gallons of milk a day. They had no recent history of mastitis and were designated as suitable animals if their total milk cell counts were below 100,000 cells/ml. of which neutrophil cell counts were only 10–15 %. Milk samples were stained by the 'single dip' method of Broadhurst & Paley (1939) and cells were counted by the technique of Pattison & Holman (1951).

Whole milk samples

Quarter milk samples from individual cows were collected in the afternoon, after an initial strip milking. They were cooled and used immediately or frozen at $-20°$ C. for subsequent work.

Milk whey samples

Whole milk was centrifuged at 82,000 ***g*** for 60 min. and the clear whey pipetted from the casein and milk lipid layers before storing at $-20°$ C.

Production of a sterile mastitis in the bovine mammary gland

The cows giving normal milk and described above were inoculated intramammarily with 1 μg. of *Escherichia coli* endotoxin. This inoculation will reliably produce a mild sterile mastitis and has been reported by Brownlie (1972).

Milk whey from experimental mycoplasma mastitis

Whey from the milk of a cow (B89) that had been inoculated with T-mycoplasma strain O13 and had mastitis at the time of sampling, was retained and stored at $-20°$ C. This whey was assayed to examine the differences in mycoplasmacidal activity of wheys from endotoxin-stimulated and mycoplasma-infected mammary glands.

Dialysis of whey samples

A variation in the mycoplasmacidal activity of whey samples was demonstrated after prolonged dialysis. Ten ml. of whey was dialysed against a barbitone buffer (complement fixation test diluent – Oxoid Ltd, London) in the 27/32 Visking dialysing tube of Scientific Supplies Company, London. The dialysis period varied from 1 h. to 24 hr. and all procedures were carried out at 4° C.

T-mycoplasma strains

The bovine strains T288, T95 and T488 were provided by Dr Livingstone (Texas, U.S.A.) whereas the remaining bovine strains were isolated at Compton. The virulence of these Compton strains has been assayed by intramammary inoculation (Howard *et al.* 1973) with the exception of strains GraX and D48.

The human strains 7, 23, 27, 58, 354, Pirillo, Cook and T960 were provided by Dr F. T. Black (Aarhus, Denmark) and have been described by him (Black, 1973). The remaining human strains CD573, CD408, CD343 and the canine strain Sp1701 and simian strain Sp1625A were supplied by Dr D. Taylor-Robinson (M.R.C. Clinical Research Centre, Northwick Park, London), and have been described previously (Howard *et al.* 1973).

Chromatographic fractionation of milk whey

Whey from cow (C14) was fractionated on Biogel A – 1·5 m. (100–200 mesh) in complement fixation test diluent. After concentration threefold in carbowax, 9 ml. of whey were applied to a column 2·5 cm × 90 cm (Pharmacia AB, Sweden) and the eluate recorded at 280 nm. The individual peaks were adjusted to 9 ml. and assayed for mycoplasmacidal activity.

The presence of immunoglobulins in these peaks was demonstrated by double diffusion plates with antisera to bovine globulins. (Antisera were kindly supplied by T. J. Newby, Department of Animal Husbandry, University of Bristol, Langford, Bristol.)

T-mycoplasmacidal activity of milk and whey samples and fractions

Overnight cultures of strains of T-mycoplasmas, grown in U2 broth (Gourlay, Brownlie & Howard, 1973) were used to determine mycoplasmacidal activity. Samples of milk, whey or fractions (1·8 ml.) were tested in duplicate by adding 0·2 ml. of culture, and the mixture was incubated at 37° C. on an orbital shaker at 120 rev./min. Duplicate 0·1 ml. samples were removed from the inoculation mixture at various times for the estimation of viable organisms and expressed as colour change units per ml. (c.c.u./ml.) (Gourlay *et al.* 1972).

RESULTS

The mycoplasmacidal activity of milk from normal and endotoxin-stimulated glands against 25 *T-mycoplasma strains*

Twenty-five T-mycoplasma strains from cattle and other animal species were incubated separately with normal bovine milk (cow A404). The viable organisms in the incubation mixture were assayed at timed intervals and results showed that killing was negligible at 1 hr. but evident at 4 hr. and more extensive at 24 hr. After 4 hr., normal milk was mycoplasmacidal to 7 of the 13 bovine strains (Table 1). The ten human strains and the canine and simian strains appear to resist any killing by normal milk over 4 hr. However, certain of the human strains Reow, CD408 and CD573 were killed within 24 hr. by the same milk sample.

Table 1. *Assay of mycoplasmacidal activity of milk from a normal and an endotoxin-stimulated mammary gland for 25 different strains of T-mycoplasmas*

Strains	Source	Virulence	Inoculum log no. viable organisms/ml.	No. of logs reduction in viable organisms/ml. after 4 hr at 37° C.	
				Normal milk	Endotoxin-stimulated milk
		SENSITIVE TO WHEY			
Vic9	Bovine lung	+ +	6·7	6·7	2·7
O13	Bovine eye	+ +	4	4	0
O12	Bovine urogenital tract	+ +	4	2	N.T.
A417	Bovine lung	+ +	5	3	1
GraX	Bovine lung	N.T.	3	3	1
T288	Bovine lung	N.T.	6	2	N.T.
		RESISTANT TO WHEY			
Bu2	Bovine urogenital tract	—	5	1	0
B101		—	6	1	1
T488		N.T.	5	0	N.T.
M525		+ +	4·7	0	N.T.
D20	Bovine lung	+	3·7	0	0
T95	Bovine lung	N.T.	6	0	N.T.
D48	Bovine lung	N.T.	4	0	N.T.
II	Human urogenital tract	N.T.	4	0	N.T.
III		N.T.	5	1	N.T.
IV		N.T.	5	0	N.T.
V		N.T.	5	0	N.T.
VII		N.T.	3	0	N.T.
Reow		—	5	0	N.T.
T960		—	5	0	N.T.
CD573		—	5	0	N.T.
CD408		—	5	0	N.T.
CD343	Human oral cavity	—	5	0	N.T.
Sp1701	Canine urogenital tract	—	5	1	0
Sp1625A	Simian throat	—	4	0	0

Virulence (Howard *et al.* 1973): + +, causes clinical mastitis; +, causes subclinical mastitis; —, avirulent; N.T. not tested.

Milk from an endotoxin-stimulated gland (cow A404) possessed reduced mycoplasmacidal activity against the 7 bovine strains killed by normal milk, and no activity against all the other strains examined (Table 1).

Comparison of mycoplasmacidal activity of whey obtained from milk of normal and endotoxin-stimulated mammary glands

The clear whey fraction obtained from milk by high-speed centrifugation contained most of the milk protein with the exception of sedimented caseins.

Assays of the mycoplasmacidal activity of normal milk and their respective wheys from 6 cattle demonstrated that whey contained an activity equal to the corresponding milk. This is particularly interesting in the case of whey from the

mastitis milk of endotoxin-stimulated glands of cows A404, D816, PL2 and C44. The activity of this whey is equal to the mastitis milk and is lower than whey from normal milk of the same cow. Similarly, the whey from milk of an experimental case of T-mycoplasma mastitis, cow M614, had a reduced activity compared with whey from its normal milk taken before inoculation.

Freezing milk and whey samples at $-20°$ C. for periods up to 3 months did not appear to alter this activity.

Comparison of mycoplasmacidal activity in the normal milk wheys from different cows

Coincident with the variation of whey mycoplasmacidal action for different T-mycoplasma strains, a cow-to-cow variation was expected. Accordingly, the wheys from five cows were incubated with five of the T-mycoplasmas, selected to include sensitive and resistant strains. The normal milk wheys from all five cows showed activity against strains Vic9 and A417, whereas against the remaining three strains Bu2, CD573 and Reow there was no demonstrable activity. The variation between cows was small. The wheys from a further 11 cows have been tested against the most sensitive of these T-mycoplasmas, strain Vic9. Only seven out of eleven gave reduction in viable count after 4 hr. incubation at 37° C., and thus a certain amount of animal variation was evident.

In order to determine heat lability of the mycoplasmacidal components whey samples from two cattle, C14 and D304, were heated to 56°, 60° and 70° C. for 60 min. Both wheys retained all activity after being heated to 56° and 60° C. for 60 min., but had their 5-log activity reduced by 2-logs of viable organisms, after heating at 70° C. for 60 min.

Fractionation of milk whey

Biogel A – 1·5 m. chromatography. Whey from cow C14 was fractionated on Biogel A – 1·5 m. Five distinct peaks A–E were isolated and of six fractionations, Fig. 1 is the typical chromatographic profile. Peak A coincided with the exclusion volume and contained excluded molecules of approximately $1{\cdot}5 \times 10^6$ daltons. The whey from the milk of three different cows C20, C30 and C14 was fractionated and gave similar chromatographic profiles to Fig. 1. All five distinct peaks following the three fractionations were assayed for mycoplasmacidal activity. Activity, equal to the original whey, was found to remain almost entirely in peak B, with reduced activity on only two occasions being detected in peak C.

Sodium dodecylsulphate (SDS) gel electrophoresis. Electrophoresis on 5% SDS polyacrylamide gel of whey and the five isolated peaks following Biogel chromatography is shown in Plate 1. From peak A towards peak E there is a progressive increase in the smaller-molecular-weight components. Peak B contains several large proteins including the immunoglobulins. Traces of these large proteins can be seen in the gels of peak A but not in the remaining three peaks C, D and E.

The presence of immunoglobulins in peak B has been demonstrated with double diffusion plates (Fig. 2). Anti-IgM serum gave a precipitin line in peaks A and B. Anti-IgG serum gave a line in both peaks B and C while Anti-IgA serum gave a line in peak B only.

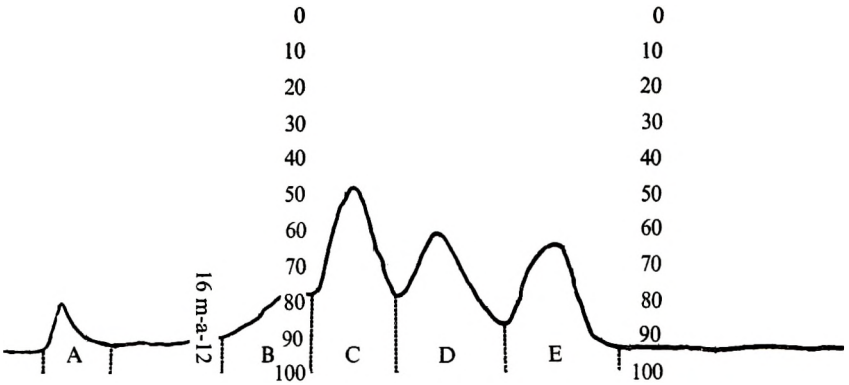
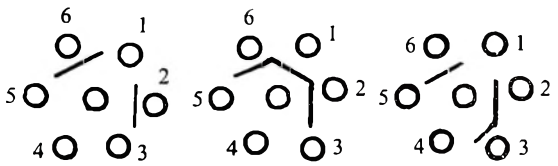


Fig. 1. Chromatographic profile at 280 nm. of whey from Cow C14 following fractionation on Bio-Gel A – 1·5 m.



Centre well	IgA	IgM	IgG
	1 Peak A	2 Peak B	3 Peak C
Peripheral wells	4 Peak D	5 Peak E	6 Whole whey

Fig. 2. Double diffusion plates of whey and whey fractions (Fig. 1) against bovine IgA, IgM and IgC, antisera.

Dialysis experiments. Dialysis of 1 volume of whey against 10 volumes of barbitone buffer resulted in no change in activity for the first two hours but, beyond that time, there was complete loss of activity. This loss of activity, following dialysis, was repeatable in eight subsequent experiments. No change was found when the knotted ends of the dialysis bag were above the solution. However, if the buffer volume for dialysis was equal to the whey volume, mycoplasmacidal activity could be demonstrated in both whey and buffer.

DISCUSSION

Certain bovine T-mycoplasma strains are capable of producing experimental mastitis in cattle whereas the human, simian and canine T-mycoplasma strains examined have had no effect when inoculated into the mammary gland (Gourlay *et al.* 1972; Howard *et al.* 1973). This *in vivo* variation in pathogenicity is probably due to both the virulence of the mycoplasmas and the activity of host local defence mechanisms. These latter mechanisms operate within the mammary gland, but little is known of their activity against mycoplasmas.

Our results show that six bovine T-mycoplasma strains, Vic9, O13, U12, A417, GraX and T288 are highly sensitive *in vitro* to both normal bovine milk and whey. However, the human, simian, canine and the remaining bovine strains in Table 1 were not so sensitive. These results do not accord with a simple explanation that the mycoplasmas which are capable of causing mastitis are more resistant to milk and whey mycoplasmacidal action. On the contrary, the virulent strains appeared to be the least resistant (Table 1).

The results also show that the mycoplasmacidal action of milk from normal glands containing less than 10^5 cells/ml. is greater than that of milk from endotoxin-stimulated glands of the same cow which contained 10^6 cells/ml. This contrasts with the results for bactericidal activity of normal milk which is always increased after pyrogen stimulation (Derbyshire, 1964). It may indicate that the mycoplasmacidal and bactericidal systems in milk are different. The reduction in mycoplasmacidal activity after endotoxin stimulation did not appear to be due to the release of active serum components in milk. These components will increase in milk because of the galactophoritis caused by endotoxin infusion (Brownlie, 1972), but addition of 30 % bovine serum to normal whey did not alter its *in vitro* activity.

One possible explanation for the reduction in activity might be the protection of mycoplasmas by milk cells. Neutrophils form over 90 % of the milk-cell population in endotoxin-stimulated milk (Brownlie, 1972) and in this context the study of mycoplasma-cell interaction by Simberkoff & Elsbach (1971) is particularly pertinent. They were unable to demonstrate killing of *Mycoplasma hominis* and *M. arthritidis* by rabbit neutrophils although the neutrophils were able to kill *Escherichia coli* and staphylococci. They suggested that neutrophils were unable to kill mycoplasma and this could explain the lack of killing in endotoxin-stimulated milk. Moreover, Lloyd & Trethewie (1970) found that mycoplasmas can survive in the presence of bovine macrophages. However, human macrophages have been shown to phagocytose and cause degradation of *M. pneumoniae*, *M. neurolyticum* and *M. gallisepticum* (Zucker-Franklin, Davidson & Thomas, 1966) and, similarly, murine macrophages are capable of phagocytosing and digesting opsonized *M. pulmonis* (Jones & Hirsch, 1971). Recently, Cole & Ward (1973) have demonstrated a protective effect by murine peritoneal macrophages of *M. arthritidis* against antibody.

An alternative explanation for the reduced activity in milk after endotoxin stimulation could be that with the increase in neutrophils there is either a concurrent increase in an inhibitor of mycoplasmacidal activity or adsorption of the active components by milk cells. Both mechanisms require the wheys from endotoxin-stimulated milk to have a reduced activity, compared with normal whey. This was shown, in the results, to be the case.

The retention of mycoplasmacidal activity in whey from normal milk, shows that soluble, and not cellular, components are responsible. Moreover, the assay of wheys from different cows demonstrated that there was some cow-to-cow variation of this soluble fraction.

The isolation of activity in peak B alone, after Biogel chromatography (Fig. 1),

indicates a molecular size of about 400,000 daltons, that of IgA. However, the SDS-polyacrylamide-gel electrophoresis of peak B (Plate 1), shows five protein-staining bands. Most of the protein is in two large molecular weight bands which are not found to any extent in the remaining four peaks. By elimination, these two bands may be associated with the mycoplasmacidal activity.

The question then arises, could the activity in milk be due to specific antibody? This would appear to be possible from the chromatographic evidence, but since mycoplasmacidal activity is not affected by heating to 60° C. for 60 min. complement is clearly not involved.

Finally, the dialysis experiment gave unexpected results. Activity was lost following the dialysis of one volume of whey in commercial Visking tubing against a tenfold volume of baritone buffer, and phosphate-buffered saline. However, the dialysis of equal volumes of whey and buffer appeared to allow an equilibrium to be established with activity in both whey and dialysing buffer. This indicates that components responsible for activity can pass through a dialysis bag. This contrasts with the chromatography where activity was only in the large-molecular-weight fraction of peak B.

One explanation of these results could be that the small active molecules aggregate and therefore chromatograph in peak B. On dialysis, there is disaggregation and the active components form an equilibrium across the membrane. Reaggregation in the dialysing buffer may occur. Alternatively the activity might be due to a small-molecular-weight component which in whey is bound to large molecules like immunoglobulins. On dialysis, the small active component separates from its carrier molecule and moves through the membrane forming an equilibrium between whey and buffer. If the volume of buffer is small, then activity can be demonstrated in the buffer with our assay technique. These dialysis results clearly distinguish this dialysable activity from the non-dialysable factor of Taylor-Robinson *et al.* (1969), from lactenin (Wilson & Rosenblum, 1952), and from lactoferrin (Oram & Reiter, 1968). It is hoped that further work will establish the nature of the mycoplasmacidal activity. Small basic proteins with non-specific antimicrobial activity have already been demonstrated (Hirsch, 1960; Brownlie & Hibbitt, 1972) and it may be a molecule of this nature that is responsible.

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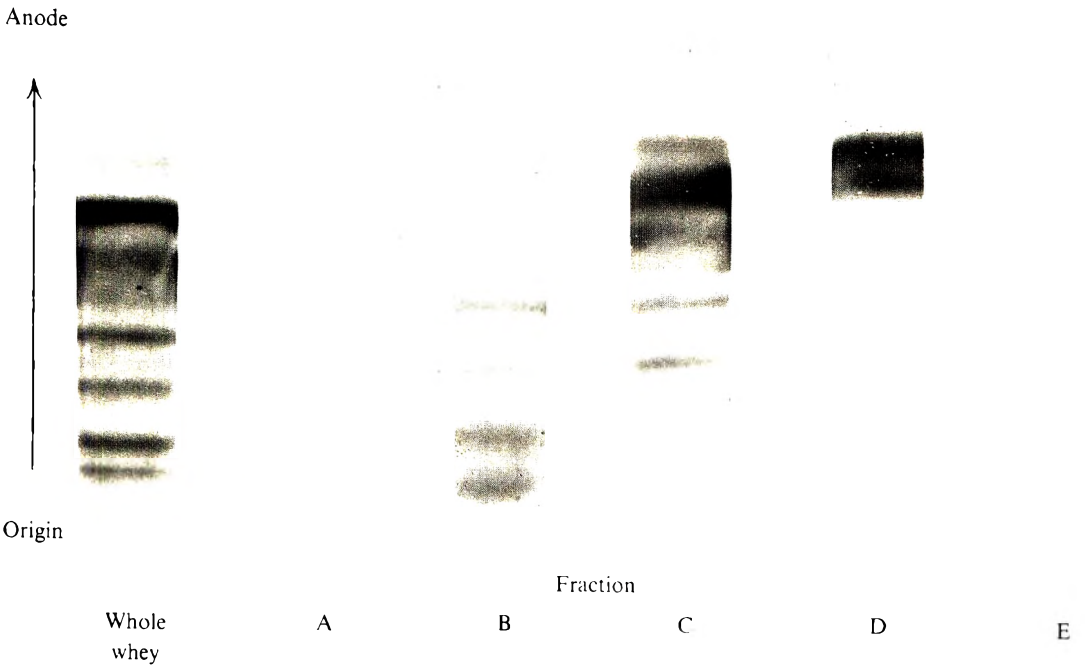

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EXPLANATION OF PLATE

SDS gel electrophoresis of whey and whey fractions (Fig. 1).

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Characterization of the antibodies detected by the microscopic agglutination test for bovine leptospirosis

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SUMMARY

The nature of the antibodies detectable by the microscopic agglutination test for bovine leptospirosis was examined. Density gradient ultracentrifugation, gel filtration and disulphide-bond-reduction experiments indicated that antileptospiral agglutinating activity was present in both IgM and IgG immunoglobulin fractions. This was confirmed by selective precipitation of specific antibody classes and ion-exchange chromatography.

INTRODUCTION

Leptospira are often difficult to isolate from infected cattle and therefore diagnosis usually depends on the detection of specific antibodies. However, the efficiency of serological diagnosis obviously depends on the nature of the immunoglobulins active in the tests employed. The Rose Bengal plate test (RBPT) used in the detection of bovine brucellosis appears to be mediated solely by immunoglobulins of the IgG_1 sub-class (Corbel, 1972). It follows that the RBPT would be ineffective in detecting an early infection in which the immunoglobulins were predominantly IgM. In contrast, Duffus & Allan (1968) found that an indirect haemagglutination test was only capable of detecting the IgM response of chickens infected with *Salmonella gallinarum* although specific IgG antibodies were detectable by other means.

The microscopic agglutination (MA) test is the recognized standard serological test for bovine leptospirosis (World Health Organization, 1967). However, despite its extensive use, the antibodies detectable by the MA test have not been examined in bovine sera. A study to identify the agglutinins evoked in cattle by leptospira was therefore desirable in order to establish the phases of the immune response measured and, hence, the validity of the MA test in diagnosis.

MATERIALS AND METHODS

Serum samples

Bovine sera. Bovine sera from 3 animals each reacting to diagnostic titres ($\geqslant$ 1/100) with *Leptospira interrogans*, serotypes *icterohaemorrhagiae* and *canicola* were provided by the leptospirosis section at this laboratory. With the exception

of the ion-exchange chromatography studies, in which each of the samples was examined separately, pooled sera were examined.

Rabbit sera. Class-specific antiglobulins to bovine IgM and IgG had been previously prepared in New Zealand White rabbits using a similar method to that described by Negi, Myers & Segre (1971*a*). The antisera were rendered specific by absorption with purified bovine IgM or IgG polymerized with gluteraldehyde (Avrameas & Ternynck, 1969).

Microscopic agglutination test

The microscopic agglutination (MA) test, using living antigen suspension, was performed as described by Wolff (1954). The antigens used consisted of serotypes *icterohaemorrhagiae* and *canicola*, grown for 7 to 10 days at 30° C. in Korthof medium. Serum fractions were tested separately against each antigen.

Disulphide-bond reduction

Serum fractions were incubated at 37° C. for 60 min. with equal volumes of aqueous 0·2 M dithiothreitol (DTT, Hopkins & Williams). Serial doubling dilutions of the mixture were prepared in 0·15 M-NaCl and tested for agglutinating activity.

Selective removal of immunoglobulins

Class-specific rabbit antibovine globulin sera were used for the selective removal of IgM and IgG from whole bovine serum. The bovine serum was added to 4 volumes of the appropriate antiglobulin preparation and incubated for 16 hr. at 37° C. The precipitate was deposited by centrifugation at 10,000 *g* for 30 min. and the absorbed serum examined by immunoelectrophoresis and agglutination.

Density gradient ultracentrifugation

Gradients of $NaNO_3$ and KBr (Cowan & Trautman, 1965) were prepared according to Corbel (1972). Samples of 2·0 ml. of serum were centrifuged at a relative centrifugal velocity of 110,000 *g* for 24 hr. at 12° C. Serum fractions were aspirated in 0·5 ml. volumes and dialysed against 0·1 M phosphate buffer at pH 7·0.

Gel filtration

Gel filtration was performed in 700 mm. × 26 mm. columns of Sephadex G200 (Pharmacia). The gels were swollen in Tris-HCl buffer (1 M-NaCl; 0·1 M Tris-HCl, pH 8·0; 0·01 M-NaN_3) and equilibrated with 2 column volumes of the Tris-HCl eluant. Sera were dialysed overnight against the column buffer and 3·0 ml. volumes were applied using a three-way valve and eluted by upward development of the eluant at a flow rate of 20 ml. per hr. Column effluent was monitored for absorption at 280 nm. using a Uvicord ultraviolet absorptiometer (LKB Produkter). The fractions were collected automatically in 5 ml. volumes using an Ultra-Rac fraction collector (LKB Produkter), dialysed as described previously and concentrated to the original volume of serum with minicon macrosolute concentrators (Amicon Ltd).

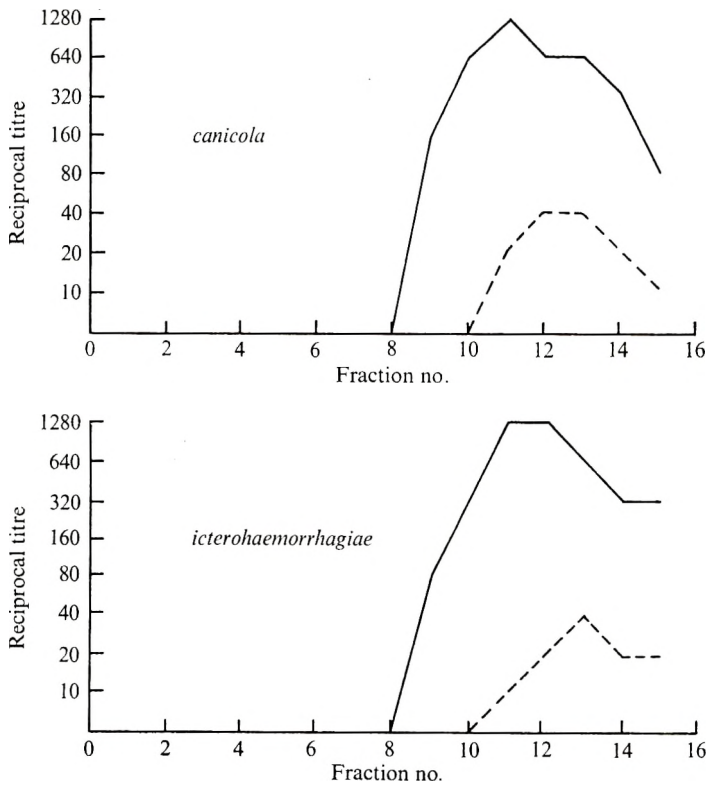

Fig. 1. Antileptospiral agglutinating activity of bovine serum separated by density gradient ultracentrifugation. ——, microscopic agglutination test (MA); – – – –, microscopic agglutination test after treatment with 0·2 M dithiothreitol (DTT).

Ion-exchange chromatography

Each of the serum samples was fractionated on QAE Sephadex A50 (Pharmacia) equilibrated with 0·1 M phosphate buffer at pH 7·0 in 300 mm. × 9 mm. columns. Serum was dialysed against the starting buffer and 2·0 ml. volumes added to the column which was developed by an increasing stepwise NaCl gradient in 0·01 M phosphate buffer at pH 7·0. Fractions of 2·0 ml. were collected and treated as described above.

Immunoelectrophoresis

Immunoelectrophoresis was performed on 82 mm. square slides in 1·5% ion agar in veronal buffer at pH 8·6 (I = 0·05) for 100 min. at a constant current of 15 mA. (Shreeve & Sojka, 1971).

RESULTS

Density gradient ultracentrifugation

The results of the density gradient ultracentrifugation are summarized in Fig. 1. Agglutinating activity to *icterohaemorrhagiae* and *canicola* was detected in both the fast-sedimenting and slowly sedimenting fractions. Reduction with DTT

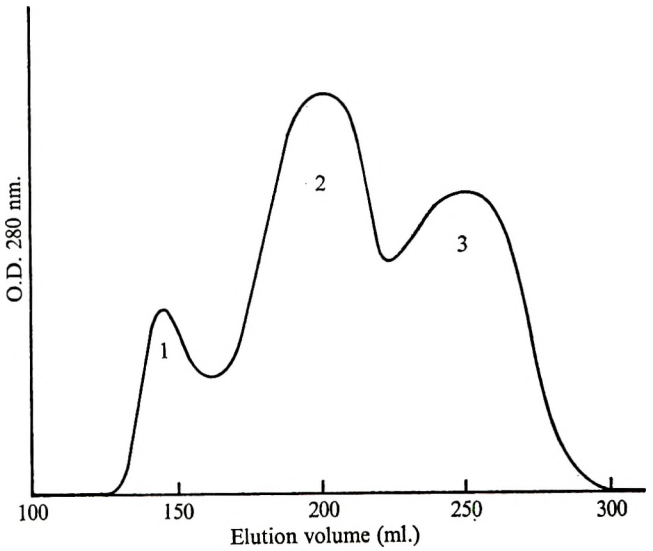

Fig. 2. Elution profile of bovine serum separated on Sephadex G200.

totally destroyed the agglutinating activity of the leading fast sedimenting fractions whereas the slower sedimenting fractions contained DTT-resistant immunoglobulins.

Gel filtration

The absorption profile of the serum separated on Sephadex G200 is shown in Fig. 2. Agglutinating activity to both serotypes was present in fractions corresponding to peak 1 and peak 2. Treatment with DTT showed that the agglutinins present in peak 1 were reduction-labile whilst DTT-stable agglutinins were eluted in peak 2 (Table 1). No agglutinating antibodies were detected in the fractions from peak 3.

Immunoglobulin inhibition

Immunoelectrophoresis of bovine serum absorbed with class-specific antiglobulin showed that the appropriate class of antibody had been successfully removed by precipitation. Microscopic agglutination tests after selective removal of IgM or IgG showed a significant reduction in the agglutination titre to *icterohaemorrhagiae* and *canicola* (Table 2). Absorption with normal rabbit serum had no effect on the titre.

Ion-exchange chromatography

Sera from each of the three animals were separated using the anionic exchange resin QAE Sephadex A50 and the eluted fractions tested for agglutinating activity against *icterohaemorrhagiae* and *canicola*. The absorption profile is shown in Fig. 3. No agglutinins were detectable in the fractions corresponding to peak 1, but DTT-resistant agglutinins were present in the fractions eluted in peak 2. The

Table 1. *Antileptospiral agglutinating activity of pooled bovine serum fractions separated by gel filtration on Sephadex* G200

Fraction no.	Serotype: *icterohaemorrhagiae* MA	*icterohaemorrhagiae* DTT	*canicola* MA	*canicola* DTT
Whole	320	80	160	40
1–25	—	—	—	—
	PEAK 1			
26	—	—	—	—
27	—	—	—	—
28	20	—	—	—
29	20	10	—	—
30	80	10	40	—
31	40	—	20	—
32	40	—	10	—
33	20	—	20	—
	PEAK 2			
34	10	—	—	—
35	—	—	—	—
36	—	—	—	—
37	20	—	10	—
38	40	20	20	10
39	40	20	20	10
40	40	40	10	—
41	10	—	10	—
42	—	—	—	—
43	—	—	—	—
	PEAK 3			
44	—	—	—	—
45	—	—	—	—
46	—	—	—	—
47	—	—	—	—
48	—	—	—	—
49	—	—	—	—
50	—	—	—	—
51	—	—	—	—
52	—	—	—	—

Titres expressed as reciprocal of final dilution showing agglutination.

MA, microscopic agglutination test; DTT, microscopic agglutination test after treatment with 0·2 M dithiothreitol; —, < 10.

majority of the DTT-labile agglutinins were eluted in peak 3 although activity was noted in some of the fractions corresponding to peak 4. No agglutinins were detected in the fractions from peak 5. Agglutinins were active against both serotypes tested (Table 3).

Immunoelectrophoresis detected only IgG_2 in the fractions eluted in peak 1. The proteins eluted in peak 2 appeared to be mainly IgG_1 with small amounts of IgG_2. IgM appeared to be the major immunoglobulin of peak 3 although traces

Table 2. *Antileptospiral agglutinating activity of bovine serum after selective precipitation of immunoglobulin classes*

Precipitating agent	Serotypes: *ictero-haemorrhagiae*	Serotypes: *canicola*
Normal rabbit serum	320	160
Rabbit anti-bovine IgM	40	40
Rabbit anti-bovine IgG	80	40

Titres expressed as reciprocal of final dilution showing agglutination.

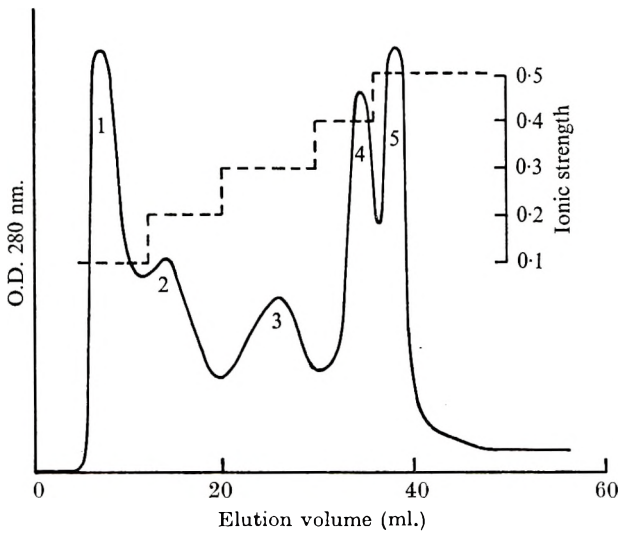

Fig. 3. Elution profile of bovine serum separated on QAE Sephadex A50.

of IgG_1 and possibly IgA were also present. The protein eluted in peaks 4 and 5 was more heterogeneous and contained only traces of γ-globulins.

DISCUSSION

There is little information concerning the nature of the immune response of cattle to leptospira infection. An examination of colostrum from infected dams suggested that while IgA exhibited neutralizing activity, agglutinating activity was associated only with IgM antibodies (Sasaki & Arima, 1971). Specific IgG antibodies were not reported. Killed vaccines appeared to evoke insignificant agglutination titres in cattle, but appreciable levels of antileptospiral immunoglobulins of the IgM and IgG classes were detected using an antiglobulin test (Negi, Myers & Segre, 1971*b*). Furthermore, it was demonstrated that both these classes of antibody imparted some degree of protection to hamsters subsequently challenged with serotype *pomona*. In a recent review of the immunology of bovine leptospirosis, Hanson (1973) concluded that antigenic stimulation results initially

Table 3. *Antileptospiral agglutinating activity of bovine serum fractions separated by ion-exchange chromatography on QAE Sephadex A50*

Fraction no.	Peak no.	Cow 10 Ict MA	Cow 10 Ict DTT	Cow 10 Can MA	Cow 10 Can DTT	Cow 17 Ict MA	Cow 17 Ict DTT	Cow 17 Can MA	Cow 17 Can DTT	Cow 18 Ict MA	Cow 18 Ict DTT	Cow 18 Can MA	Cow 18 Can DTT
Whole		640	60	320	40	640	80	320	40	320	80	160	40
1	1	—	—	—	—	—	—	—	—	—	—	—	—
2		—	—	—	—	—	—	—	—	—	—	—	—
3		—	—	—	—	—	—	—	—	—	—	—	—
4		—	—	—	—	—	—	—	—	—	—	—	—
5		—	—	—	—	—	—	—	—	—	—	—	—
6	2	20	20	—	—	10	10	20	10	20	10	10	—
7		40	20	20	10	40	20	20	10	20	20	40	40
8		40	20	40	20	20	20	10	10	80	40	40	20
9		20	10	20	10	—	—	—	—	80	40	20	40
10	3	10	—	10	—	10	—	—	—	40	20	—	—
11		10	—	10	—	10	—	—	—	10	—	—	—
12		20	—	—	—	10	—	—	—	—	—	20	—
13		80	—	40	—	10	—	20	—	20	—	—	—
14		160	20	40	—	80	—	20	—	20	—	10	—
15		40	—	20	—	160	—	80	10	80	10	80	—
16	4	20	—	10	—	40	—	40	—	40	—	40	—
17		—	—	—	—	20	—	—	—	20	—	20	—
18		—	—	—	—	10	—	—	—	10	—	—	—
19	5	—	—	—	—	—	—	—	—	—	—	—	—
20		—	—	—	—	—	—	—	—	—	—	—	—
21		—	—	—	—	—	—	—	—	—	—	—	—
22		—	—	—	—	—	—	—	—	—	—	—	—

Ict, *icterohaemorrhagiae*; Can, *canicola*. For other notes see Table 1.

in a relatively short period of IgM production, associated with agglutination reactions, and later stimulation of neutralizing IgG antibodies detectable for a considerable time by hamster protection tests. If this is correct, the microscopic agglutination test might be expected to detect only the initial period of leptospira infection.

The association of agglutinating activity with IgM antibodies was confirmed in the present study. Fast-sedimenting immunoglobulins with agglutinating activity were demonstrated by density gradient centrifugation. The agglutinating activity in the fractions containing this class of immunoglobulin was destroyed by reduction with dithiothreitol (DTT) but similar activity was also demonstrated in the slow-sedimenting DTT-stable antibody, suggesting that both 19S (IgM) and 7S (IgG) agglutinins were present in the sera. Gel filtrations on Sephadex G200 confirmed that specific leptospiral activity was present in reduction-labile macroglobulin fractions and DTT-resistant fractions of lower molecular weight. Selective removal of IgM or IgG using class-specific antiglobulin sera caused a significant reduction in titre thereby indicating that both classes of antibody contributed to the antileptospiral agglutinating activity of the whole serum. This conclusion was strongly supported by ion-exchange chromatography on each of the serum

samples. Immunoelectrophoresis indicated that the γ-globulins were resolved into an IgG_2 fraction, a fraction containing IgG_1 and IgG_2, and a fraction comprising mainly IgM (and possibly some IgA). No agglutinating activity was detected in the IgG_2 fraction, but each of the IgG_1- and IgM-containing fractions were active in the MA test.

These results indicate that antileptospiral agglutinins are not restricted to the IgM class but that IgG_1 antibodies also exhibit agglutinating activity. Since the synthesis of IgG is said to lag behind that of IgM, although reaching higher concentrations and persisting appreciably longer than IgM, the microscopic agglutination test may detect residual antibodies over a far longer period than earlier studies of cattle sera suggest.

The technical assistance of Mrs J. E. Gill is gratefully acknowledged.

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The survival and growth of *Bacillus cereus* in boiled and fried rice in relation to outbreaks of food poisoning

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SUMMARY

A number of outbreaks of food poisoning attributed to *Bacillus cereus* have been reported recently and all have been associated with cooked rice usually from Chinese restaurants and 'take-away' shops.

Tests were made to assess the heat resistance of *B. cereus* spores in aqueous suspension, the growth of the organism in boiled rice stored at temperatures in the range 4–55° C., and the effect of cooking and storage on the growth of the organism in boiled and fried rice. The spores of *B. cereus* survived cooking and were capable of germination and outgrowth. The optimum temperature for growth in boiled rice was between 30° and 37° C. and growth also occurred during storage at 15° and 43° C.

To prevent further outbreaks it is suggested that rice should be boiled in smaller quantities on several occasions during the day, thereby reducing the storage time before frying. After boiling the rice should either be kept hot (> 63° C.) or cooled quickly and transferred to a refrigerator within 2 hr. of cooking. Boiled or fried rice must not be stored under warm conditions especially in the range 15–50° C.

INTRODUCTION

Over 30 separate incidents of food poisoning associated with cooked rice (usually fried) and usually from Chinese restaurants or 'take-away' shops have been reported in Great Britain since 1971 (Public Health Laboratory Service, 1972, 1973, and unpublished information). Bacteriological examination of food remnants and faecal specimens has failed to yield any of the organisms usually associated with food poisoning, and staphylococcal enterotoxin was not detected in three samples of cooked rice from separate outbreaks. In most of the incidents large numbers of aerobic, spore-forming bacilli, identified as *Bacillus cereus*, have been isolated from remnants of cooked rice or from faecal specimens or from both. Plate counts on blood agar of *B. cereus* in cooked rice from 17 incidents have ranged from 3×10^5 to 2×10^9/g. with a median value of 5×10^7/g. (Gilbert & Taylor, to be published).

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Table 1. *Origin of* B. cereus *cultures*

Culture	Origin
BC 1	Stock culture, NCTC 9947
BC 2	Fried rice – food poisoning outbreak 1 in England
BC 8	Fried rice – routine food sample
BC 9	Uncooked rice – routine food sample
BC 10	Fried rice – routine food sample
BC 23	Faeces – food poisoning outbreak 2 in England
BC 25	Faeces – food poisoning outbreak 3 in England
BC 40	Vanilla sauce – food poisoning outbreak in Norway
BC Br	Stock culture

Each episode has been characterized by an acute attack of nausea and vomiting usually between 1 and 5 hr. after the meal; diarrhoea has not been a common feature, occurring in only about 25 % of patients. Similar outbreaks with respect to incubation period, symptoms, food vehicles and the isolation of large numbers of *B. cereus* have been reported recently from Australia (Dr J. Taplin, personal communication) and Canada (Lefebvre, Gregoire, Brabant & Todd, 1973).

The aims of the present work were to study (i) the heat resistance of *B. cereus* spores in aqueous suspension, (ii) the growth of *B. cereus* in boiled rice stored at various temperatures, (iii) the effect of cooking and storage on the growth of the organism in boiled and fried rice; and (iv) to suggest ways to reduce or prevent this type of food poisoning.

MATERIALS AND METHODS

Bacteria

The cultures of *B. cereus* used and their sources are given in Table 1. Culture BC 2 was kindly donated by Dr P. N. Coleman, Friarage Hospital, Northallerton, BC 23 and 25 by Dr P. R. Mortimer, Public Health Laboratory, Teesside, BC 40 by Professor S. Hauge, Institutt for Næringsmiddelhygiene, Oslo 1, Norway, and BC Br by Dr A. Briggs, School of Pharmacy, University of London.

Preparation of spore suspensions

The sporulation medium was soil-extract agar (Gordon & Smith, 1955) with incubation at 30° C. for 10 days (sporulation medium 1). For two cultures, BC 9 and 25, spores were also produced on nutrient broth containing 1·5 % of Davis agar and 0·001 % of added Mn^{2+} as $MnSO_4$ and 0·001 % of added Ca^{2+} as $CaCl_2$ with incubation at 30° C. for 10 days (sporulation medium 2) or at 37° C. for 4 days (sporulation medium 3).

Vegetative cells were removed by washing 3 times with sterile distilled water and separating by differential centrifugation (Long & Williams, 1958). Aqueous spore suspensions, all > 95 % and usually > 99 % phase-bright spores, were stored at 4° C. A heat-shock treatment was not given during the preparation of spore suspensions.

Rice

Long grain rice was purchased for the work.

Diluent and colony plate count method

All dilutions were made in quarter-strength Ringer's solution.

Colony plate counts were made on blood agar containing 5 % defibrinated horse blood by means of a modified Miles & Misra (1938) technique with incubation for 18–40 hr. at 30° C. In experiments where low numbers of *B. cereus* were expected 0·5 ml. volumes were plated.

Determination of heat resistance of B. cereus *spores in aqueous suspension*

Samples (0·2 ml.) were distributed from a 1 ml. syringe into 2 ml. freeze-drying ampoules which were sealed under air. The ampoules were heated at 90°, 92·5° and 95° C. by total immersion in a thermostatically controlled water bath and at 100° C. in boiling water. Ampoules were removed at appropriate time intervals and immediately cooled in an ice–water mixture. They were opened, the contents washed out and tenfold dilutions prepared and plated.

Survivor curves of log percentage surviving organisms against time were constructed, using the mean count from 2 or 3 unheated ampoules as 100 %. Decimal reduction times (D), the time required to reduce the number of surviving organisms by 90 % at a constant temperature, were calculated from regression analyses of $\log_{10}$ colony plate counts for various intervals of time, using the digital computer program of Navani, Scholefield & Kibby (1970). Values for z, the number of degrees of temperature to bring about a tenfold change in D values, were also calculated.

Growth of B. cereus *in boiled rice*

Two-lb. (*ca.* 900 g.) quantities of rice were rinsed twice in cold water to remove superfluous starch. The washed rice was mixed with four pints (*ca.* 2270 ml.) of cold water, brought to the boil and allowed to simmer with occasional stirring until all the water was absorbed, *ca.* 20 min. The boiled rice was rinsed once in boiling water to facilitate separation of the grains and 10 g. samples were distributed into 1 lb. screw-capped jars.

Tenfold dilutions of spore suspensions of BC 2, 9 and 25 were prepared and 0·2 ml. volumes were distributed onto the surface of the rice with a 50 drop/ml. pipette to give an initial inoculum of *B. cereus* spores between *ca.* 10 and 2×10^4/g. of rice. Sets of jars were stored at 4°, 10°, 15°, 22°, 30°, 37°, 43° and 55° C. for periods of time up to 3 days. Jars were removed at various time intervals and 90 ml. of diluent were added to each to give a 1/10 dilution. After thorough mixing, further tenfold dilutions were prepared and plated on blood agar for counts.

Effect of cooking and storage on the growth of B. cereus *in boiled and fried rice*

A 5 lb. quantity of rice was rinsed twice in water and inoculated with low numbers of spores of BC 2, *ca.* 140 spores/g. or BC 9, *ca.* 680 spores/g. Ten pints of

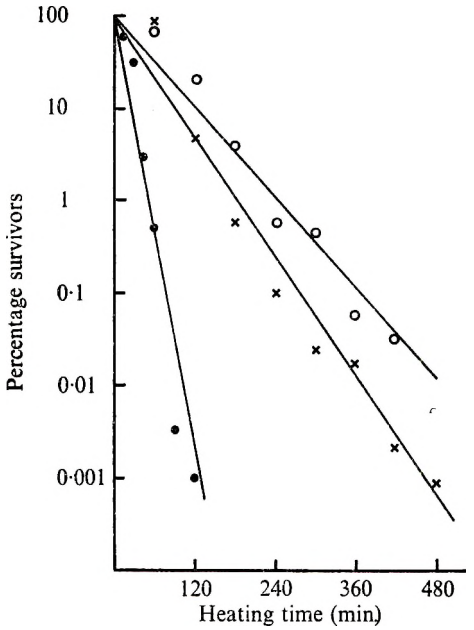

Fig. 1. Heat resistance of *B. cereus* spores in aqueous suspension at 90° C. ○, culture BC 8; ●, culture BC 9; ×, culture BC 25.

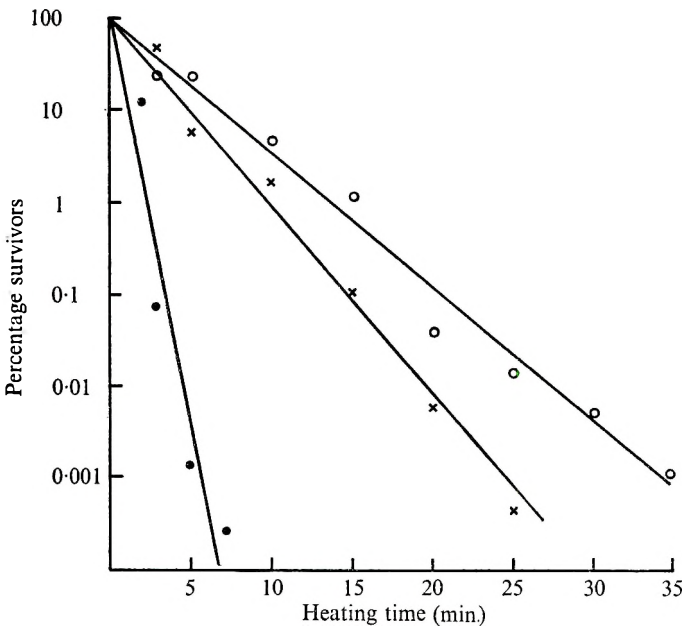

Fig. 2. Heat resistance of *B. cereus* spores in aqueous suspension at 100° C. ○, culture BC 8; ●, culture BC 9; ×, culture BC 25.

Table 2. *Heat resistance of* B. cereus *spores in aqueous suspension*

Culture	Decimal reduction times (min) at °C. 90	92·5	95	100	*z* value (°C.)
BC 1	67	36	17	2·3	6·8
BC 2	100	62	21	6·0	7·7
BC 8	112	74	36	7·5	8·3
BC 9	21	13	5·0	1·2	7·9
BC 10	93	59	24	4·2	7·2
BC 23	104	71	33	5·0	7·4
BC 25	90	57	23	4·7	7·6
BC 40	35	23	7·2	1·6	7·1
BC Br	137	73	36	4·5	6·7

water were added and the mixture cooked as before. After rinsing once with boiling water three 10 g. samples were taken from different areas (surface, middle and base) and dilutions prepared and plated. Counts were also carried out on 10 g. portions of rice, sampled at three different areas within the bulk, at intervals up to 24 hr. after boiling. During this period the cooking vessel was left on the bench to cool slowly to room temperature (23–26° C.).

A further three 250 g. portions of rice were sampled as before, and each was fried in a pan for *ca.* 1½ min. mixed with freshly beaten egg and a small amount of corn oil. Three 10 g. samples of each portion of fried rice were taken for plate counts immediately after frying and again after 24 hr. storage at room temperature. The fried rice was then fried a second time in small amounts of corn oil for *ca.* 1 min. Counts were carried out on three 10 g. samples of each portion immediately after refrying and again after 24 hr. storage at room temperature.

RESULTS

Nearly all the survivor curves for the nine *B. cereus* strains were linear on a plot of log percentage survivors against time of heating (Figs. 1, 2) with correlation coefficients greater than tabulated values at $P = 0{\cdot}05$ for the appropriate degrees of freedom. These curves are therefore exponential. Calculated D and z values are given in Table 2. The D values were in the range 21–137 min. at 90° C. and 1·2 to 7·5 min at 100° C. Of the nine strains spores of BC Br were the most resistant at 90° C. and those of BC 8 at 92·5° and 100° C. The z values were in the range 6·7–8·3° C., with a mean of 7·4° C.

Survivor curves for BC 23 and BC Br at 95° C. did not appear to be linear when plotted, and the curve for BC 2 at 95° C. showed an initial shoulder before it became exponential (Fig. 3). However, coefficients of linear correlation for these three curves were greater than tabulated values and the data could therefore be represented approximately by straight lines.

Table 3 shows D and z values for spores of BC 9 and BC 25 produced under varying conditions of growth and sporulation. For both strains the spores produced on soil-extract agar were the most resistant at 100° C.

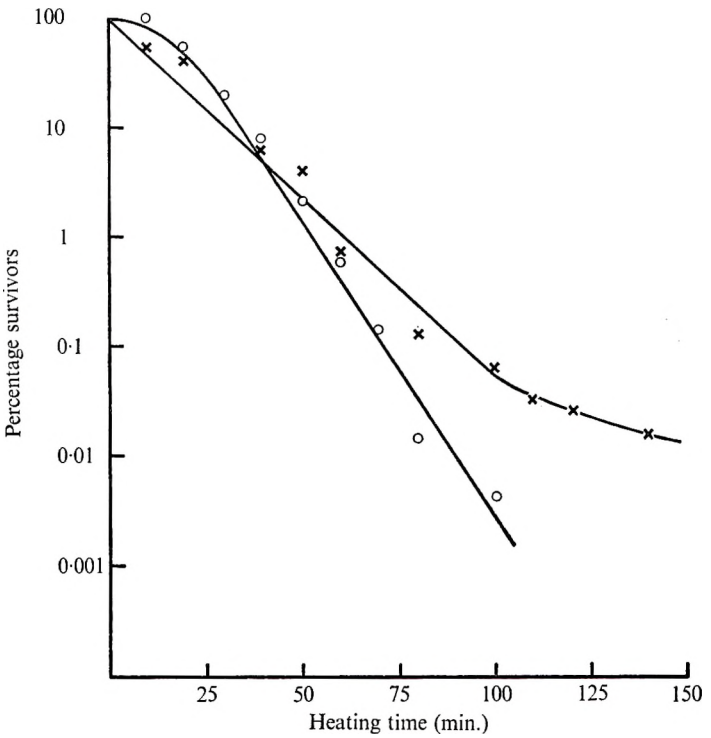

Fig. 3. Heat resistance of *B. cereus* spores in aqueous suspension at 95° C. ○, culture BC 2; ×, culture BC 23.

Table 3. *Heat resistance in aqueous suspension of* B. cereus *spores produced under different conditions*

Culture	Sporulation medium and conditions*	Decimal reduction times (min) at °C. 90	92·5	95	100	*z* value (°C.)
BC 9	1	21	13	5·0	1·2	7·9
	2	27	N.T.	4·8	0·6	6·1
	3	27	N.T.	5·2	0·9	6·8
BC 25	1	90	57	23	4·7	7·6
	2	64	N.T.	20	3·2	7·7
	3	77	N.T.	25	3·9	7·7

* See text. N.T., not tested.

Total colony counts of the uncooked rice were < 100/g. but *B. cereus* was isolated after enrichment in nutrient broth. *B. cereus* was not isolated, however, from *ca.* 2 g. of uninoculated rice taken from a large bulk which had been boiled for 20 min.

The growth of BC 2, 9 and 25 in boiled rice stored at different temperatures in the range 4–55° C. is shown in Tables 4 and 5. At 4° and 10° C. there was no growth with any of the three strains after 3 days storage. There was slight growth at 15° C., much more at 22° C. and the optimum was reached at 30° to 37° C. Growth at 43° C. was more rapid than at 15° C. but less than that at 22° C. At

Table 4. *Growth of* B. cereus *spores in boiled rice stored at* 4°, 10°, 15°, 43° *and* 55° *C.*

Culture	Storage temperature (°C.)	Log count of *B. cereus* in boiled rice after storage (hr.) 0	16	24	48	72
BC 2	4	3·74	N.T.	3·70	3·74	3·60
	10	3·74	N.T.	3·78	3·85	3·74
	15	2·74	2·78	2·88	3·18	3·30
	43	2·54	3·70	4·40	4·54	N.T.
	55	3·54	2·90	2·30	< 1·30	N.T.
BC 9	4	3·81	N.T.	3·78	3·85	3·78
	10	3·81	N.T.	3·85	3·78	3·78
	15	2·81	3·08	3·18	3·30	3·60
	43	3·30	4·18	4·65	4·74	N.T.
	55	4·30	3·48	3·24	2·30	N.T.
BC 25	4	3·60	N.T.	3·60	3·48	3·54
	10	3·60	N.T.	3·60	3·54	3·70
	15	2·60	2·78	2·88	3·18	3·30
	43	2·93	3·48	3·66	4·18	N.T.
	55	3·93	2·90	2·60	1·90	N.T.

N.T., not tested.

Table 5. *Growth of* B. cereus *in boiled rice stored at* 22°, 30° *and* 37° *C.*

Culture	Storage temperature (°C.)	Log count of *B. cereus* in boiled rice after storage (hr.) 0	4	9	18	23	28	33
BC 2	22	1·00	N.T.	3·18	5·40	5·93	6·24	6·30
	30	1·00	2·35	5·70	6·81	7·18	7·60	7·81
	37	1·00	3·40	5·40	7·30	7·54	7·78	7·85
BC 9	22	1·60	2·40	3·65	5·60	6·54	6·74	6·88
	30	1·60	2·70	6·18	7·51	7·93	8·18	8·48
	37	1·60	3·90	6·90	7·93	8·24	8·48	8·60
BC 25	22	1·30	N.T.	3·30	5·48	6·40	6·60	6·74
	30	1·30	2·70	5·65	6·70	7·00	7·48	7·60
	37	1·30	3·52	5·18	7·24	7·81	7·93	8·00

55° C. there was no growth and the number of organisms recovered decreased with storage time. Mean generation times in the logarithmic phase of growth, calculated (Rose, 1968) from plate counts of *B. cereus* in boiled rice after storage for 4 and 9 hr., were in the range 26–31 min. at 30° C. and 30–54 min. at 37° C. Total colony counts on uninoculated freshly boiled rice stored at 30° and 37° C. for 24 hr. were $2{\cdot}4 \times 10^3$/g. and $3{\cdot}5 \times 10^3$/g. respectively.

The effects of the boiling, storage, frying and storage sequence on the survival and growth of BC 2 and 9 are shown in Figs. 4–5. Fig. 4 shows the range of counts of *B. cereus* from 3 sampling sites within the rice and indicates an uneven distribution of organisms which most probably occurred when the rice was inoculated. In general, plate counts on boiled rice were greater for samples taken from the surface than for those taken from the middle or base of the rice bulk.

Counts of BC 2 and 9 were < 20/g. in inoculated boiled rice sampled immediately

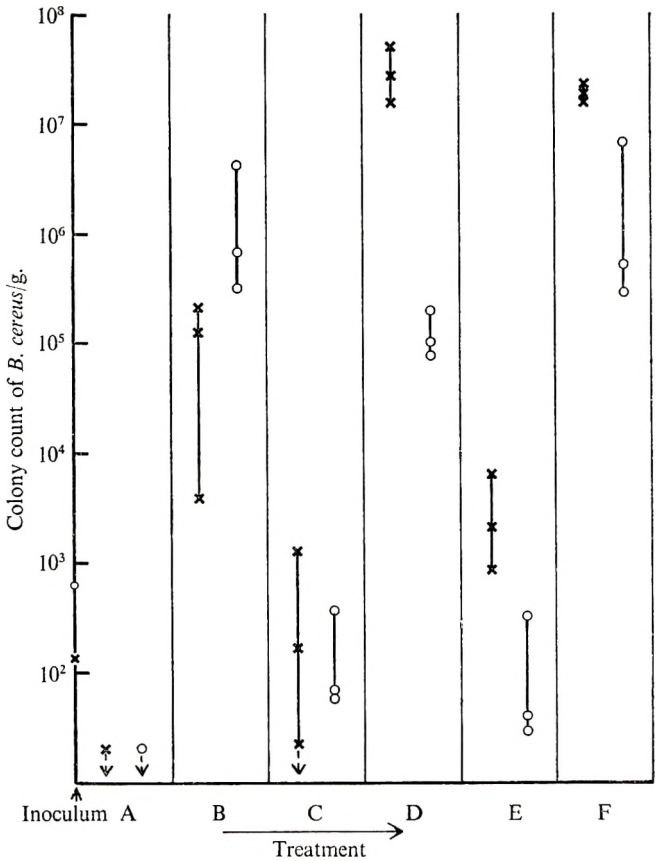

Fig. 4. Range of counts of *B. cereus* in boiled and fried rice after cooking and storage. ×, culture BC 2; ○, culture BC 9.

Key	Plate count after	Cumulative time (hr.)
A	Boiling (25 min.)	0
B	Storage	24
C	Frying (1½ min.)	24
D	Storage	48
E	Frying (1 min.)	48
F	Storage	72

after preparation and after storage for 2 hr. Viable spores were present in the rice, however, because vegetative growth occurred on subsequent storage (Fig. 5). After 24 hr. storage large numbers of *B. cereus* were present in the boiled rice and microscopic examination showed that many of the vegetative cells contained spores. Rapid frying reduced the number of organisms but those that survived, presumably heat-resistant spores, were capable of germination and outgrowth. Growth and spore formation occurred when the fried rice was stored again at room temperature. There was a second rapid fall in bacterial numbers when the rice was refried and subsequent storage for a third time again encouraged bacterial growth.

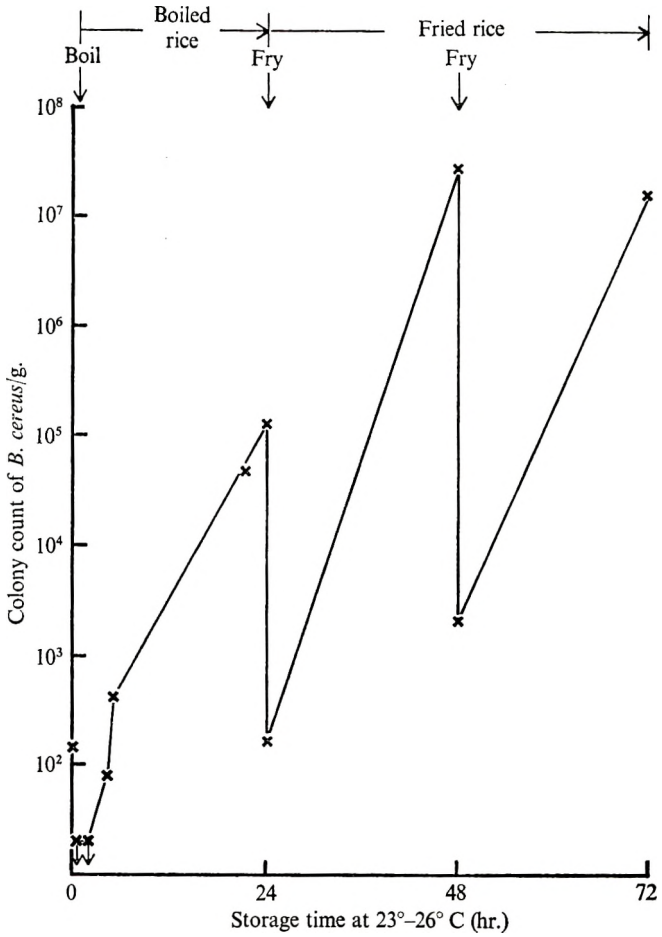

Fig. 5. Effect of cooking and storage on the growth of *B. cereus* (BC 2) in boiled and fried rice. Points plotted are median values of counts on 3 samples of rice.

DISCUSSION

The experiments described in this paper were designed to simulate the times and methods of cooking and the storage conditions used by some Chinese restaurateurs (Perry, 1974; Mortimer & McCann, 1974).

It appears to be the practice in many Chinese restaurants and 'take-away' shops to save portions of boiled rice from bulk cooking until required for frying. The boiled rice is allowed to 'dry off' at room temperature for varying periods of time from a few hours to about 3 days, but usually overnight. The rice is then either reheated or more usually fried for a very short time with beaten egg and a small amount of oil before serving: the beaten egg is not always freshly prepared and may itself be highly contaminated with a variety of bacteria. The Chinese are reluctant to store boiled rice in a refrigerator because they say the rice grains stick together and it becomes difficult to 'toss them' in beaten egg during frying. In some instances the fried rice is stored at room temperature and 'flash' fried again before serving.

The situation is made worse by the preparation of large bulks of boiled rice which take several hours to cool down, and there are reports (Mortimer & McCann, 1974) of the practice of adding fresh batches of boiled rice to the remains of old, which are sometimes left over from the previous day. Whether the boiled rice is allowed to dry off for varying periods of time at kitchen temperature or is left in or near a warm oven, conditions may be ideal for the germination and outgrowth of spores which have survived the boiling process.

Bacillus cereus is common in soil and on vegetation and has been isolated in several countries from a wide variety of routine samples of food (Nygren, 1962; Jantea, Milosescu, Bistriceanu & Bad-Oprisescu, 1965; Mossel, Koopman & Jongerius, 1967; Kim & Goepfert, 1971). Nygren (1962), for example, reported an isolation rate of 47·8% after examination of 3,888 samples of food and food ingredients in Sweden. In food poisoning outbreaks in this country the most likely source of *B. cereus* is the uncooked rice. The heat resistance of *B. cereus* during the boiling, frying or reheating of rice is important and the data presented show that organisms, presumably spores, survive cooking and are capable of germination and outgrowth.

The D100° values of the nine spore suspensions of *B. cereus* studied were in the range 1·2–7·5 min., similar to those reported by Murrell & Warth (1965) 0·8–14·2 min., Briggs (1966) 5·5 min. and Molin & Snygg (1967) 8·0 min., for spores heated in aqueous suspension or phosphate buffer. In the present study variations in sporulation medium and incubation conditions had little effect on the heat resistance of the spores produced and none of the spore suspensions showed any evidence of exceptional resistance to heat.

Results from growth experiments in boiled rice inoculated with spore suspensions of *B. cereus* (BC 2, 9 or 25) showed that the optimum temperature for vegetative cell growth was between 30° and 37° C. The minimum temperature for vegetative growth was between 10° and 15° C. Mol (1957) reported that *B. cereus* would grow in yeast extract phosphate broth when stored at 12° C. for a few days but not at 8° C. when held as long as 4 months.

The outbreaks of food poisoning attributed to *B. cereus* in Great Britain since 1971 differ in a number of respects such as incubation period, symptoms and food vehicles, from outbreaks hitherto reported in several other countries (Hauge, 1950, 1955; Goepfert, Spira & Kim, 1972). The short incubation period (between 1 and 5 hr.) for the episodes in this country is of particular interest and it suggests that the illness is caused by a toxin produced in the rice. The large numbers of *B. cereus* isolated from samples of fried or boiled rice implicated in outbreaks indicates that neither the organisms (spores) nor the toxin are destroyed in the process of frying or reheating. Work is in progress on the typing of *B. cereus* using antisera produced in rabbits against H antigens.

Until the methods described in this paper for the preparation and in particular the storage of cooked rice are discontinued, outbreaks of food poisoning will occur. Long, slow cooling and non-refrigerated storage of cooked rice, indeed of all cooked foods, provide ideal conditions for bacterial growth particularly from surviving spores. To prevent further outbreaks:

(1) Rice should be boiled in smaller quantities on several occasions during the day, thereby reducing the storage time before frying.

(2) After boiling the rice should either be kept hot, at not less than 63 °C. (145° F.), or cooled quickly and transferred to a refrigerator within 2 hr. of cooking. The cooling of rice, especially large bulks of boiled rice, will be hastened by dividing the product into separate portions or by spreading the bulk in clean shallow containers.

(3) Boiled or fried rice must not be stored under warm conditions and never at a temperature between 15° and 50° C. Under no circumstances, therefore, should cooked rice be stored at kitchen temperature for more than 2 hr.

(4) The beaten egg used in the preparation of fried rice should be freshly prepared.

We are grateful to Dr T. A. Roberts, Meat Research Institute, Langford, Bristol, for undertaking the computer analysis of results from the heat-resistance studies and to Dr Betty C. Hobbs for her advice and encouragement. We are also indebted to the many Public Health Inspectors who supplied us with information on Chinese restaurants and their culinary procedures.

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Epidemic vomiting

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SUMMARY

Two outbreaks of epidemic vomiting are described. One affected 107 students and staff at a college of education out of a total of 398 persons. The other affected 172 pupils and staff out of 357 at a secondary school. Evidence is presented that in both cases infection was acquired in the dining hall of the institution concerned but no specific item of food was found as a likely cause. The literature is reviewed. Possible mechanisms of spread are suggested.

FIRST OUTBREAK

This occurred in May 1969, and 107 students and staff were affected. During the night of 10–11 May 74 cases occurred.

The college at that time had 320 students, all female, 36 teaching and administrative staff and 42 domestic staff. One hundred and eighty-four of the students and 5 of the staff were resident as follows.

Residence A	78 students	3 staff
Residence B	18 students	1 staff
Residence C	18 students	1 staff
Residence D	18 students	
Residence E	36 students	
Approved lodgings	16 students	

Residence A has the main dining room for the college and residents have breakfast, lunch and supper. Residents in B and C have lunch and supper at A. Residents of D and E and those in approved lodgings usually have lunch at A on weekdays as also do 15–20 of the teaching and administrative staff. The domestic staff are mainly part-time and employed in the residences. About two-thirds have lunch at Residence A.

Incidence

In Fig. 1 the onset is given at 6-hourly intervals according to whether or not meals were taken at Residence A.

Table 1 gives the incidence according to Residence and Table 2 gives the incidence according to meals taken at Residence A. Of the 107 persons affected

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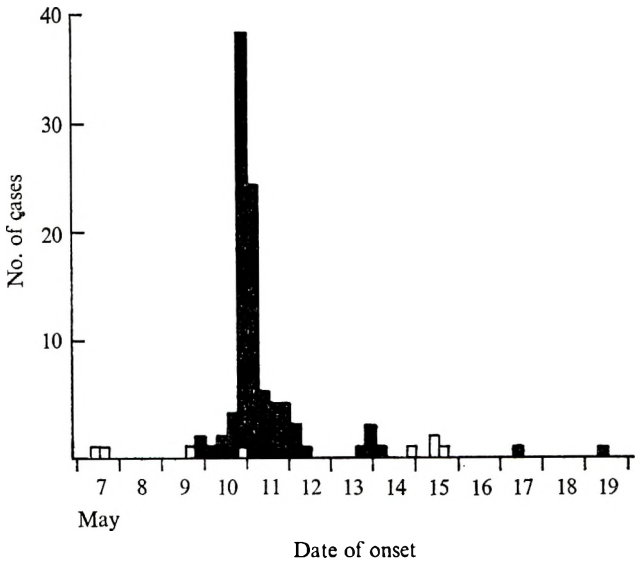

Fig. 1. Onset of cases in first outbreak, at 6-hourly intervals. □, No meal at Residence A; ■, meals at Residence A.

Table 1. *Incidence according to residence (first outbreak)*

	Total	No. affected	% affected
Students			
Residence A	78	46	60·3
Residences B and C	36	17	47·2
Residences D and E and approved lodgings	70	17	24·3
Non-resident	136	7	5·1
Staff			
Resident	5	2	40·0
Non-resident. Lunch on 9 May	15	13	86·6
Non-resident. No lunch on 9 May	16	1	6·3
Domestics			
Lunch on 9 May	28	4	14·3
No lunch on 9 May	14	—	—
	398	107	26·9

only 8 did not have meals at Residence A and of the 93 affected in the main outbreak only one did not have meals at Residence A in the previous 48 hr. Seventy-five of these had lunch on 9 May and this was the last meal which staff members and girls in Residences D, E and approved lodgings had before the outbreak started some 30 hr. later. In all 185 persons had this meal.

The heaviest incidence (87 %) was in the staff who had lunch on 9 May. On the other hand only 4 out of 28 domestics who had the same meal were affected and these 4 were all working in the kitchen. (The students and staff have two sittings and the domestics come in later.) The incidence in the students shows a progressive fall as their association with the dining room at Residence A diminishes.

Table 2. *Incidence according to meals taken at Residence A (first outbreak)*

	Meals at Residence A	No meals at Residence A	Total
Affected between 6 p.m. 9 May and midday 12 May	92	1	93
Affected other times	7	7	14
Not affected	133	158	291
Total	232	166	398

$\chi^2 = 81{\cdot}01$; $P < 0{\cdot}0005$.

Table 3. *Symptoms (first outbreak)*

	Staff		Students		Total	
	No.	%	No.	%	No.	%
Total numbers	16		65		81	
Nausea	8	50·0	59	90·8	67	82·7
Vomiting	7	43·8	57	87·7	64	79·0
Abdominal pain	5	31·2	32	49·2	37	45·7
Diarrhoea	11	68·7	26	40·0	37	45·7
Headache	2	12·5	11	18·5	13	16·0
Fainting	—		5	7·7	5	6·2
Malaise/fever/sweating	2		2		4	
Giddiness	—		2		2	
Weakness	2		—		2	

Three cases occurred before the main outbreak and there were 11 late cases. A similar type of illness was known to be prevalent in the district at the time.

The most interesting feature of this outbreak is that although persons visiting Residence A over the weekend had the impression of mass hysteria yet cases occurred simultaneously in 4 other residences and among staff and students at home or in lodgings.

Symptoms

Questionaries were not used. The number of cases, time of onset and main symptoms were ascertained by the college authorities. The 16 staff and 65 of the students were questioned personally. This was a total of 81 or 76 % of those affected. The frequency of the symptoms is given in Table 3.

Among the students nausea and vomiting were most common while among the staff diarrhoea was the leading symptom. About half of those who vomited had preliminary nausea for 1–2 hr. but the remainder had precipitate vomiting, often occurring in the early hours of the morning. The vomiting was usually succeeded by colic and diarrhoea. Thereafter recovery was very rapid. Many described their symptoms as being very severe and yet were fit and well 24 hr. later.

Other symptoms were not prominent. Five students fainted at the outset. Eleven had headaches. In one case headache, stiff neck and vertigo were the leading symptoms.

Two cases who were felt to be part of the main outbreak complained of nausea on the evening of 9 May. They vomited 24 hr. later and thereafter the illness followed the usual pattern.

Incubation period

The cases after the main outbreak provide useful clues.

(1) Five students who were away for the weekend, leaving on the morning of Friday 9 May and returning on the Sunday evening, became ill as follows:

Nausea	Vomiting
Tuesday a.m.	Tuesday midnight
Tuesday 6 pm.	Wednesday 12.30 a.m.
Tuesday 6.30 p.m.	Wednesday 2 a.m.
Tuesday 11 p.m.	Wednesday 2 a.m.
Wednesday 11 a.m.	—

This suggests an incubation period of less than 48 hr. in at least four cases.

(2) The college was closed on Monday 12 May and Tuesday 13 May because of the outbreak. It reopened on 14 May at 9 a.m. Four day-students became ill at the following times: Wednesday late p.m., Thursday a.m., Thursday a.m., Thursday p.m.

This suggests an incubation period of about 24 hr.

(3) A girl at Residence D developed typical symptoms on 17 May. Her room-mate developed the same symptoms 48 hr. later.

(4) There were only two secondary cases in the families of staff who were ill at home. Both occurred about 48 hr. after the commencement of the index case.

(5) If one now goes back to the main outbreak and assumes that the 75 persons who had lunch on the Friday and subsequently became ill were infected at the time of this meal then the incubation period would be 12 to 66 hr. with a mode of 36 hr. The majority of those having lunch on 9 May also had lunch on the previous 4 days, but a party of students were in London for 4 days, returning on the morning of 9 May. The majority of these were ill.

Investigation

Two specimens of vomit and 12 of faeces were sent to the Public Health Laboratory. Neither specimen of vomit revealed any growth on culture.

Of the 12 specimens of faeces 1 was positive for *Salmonella*, the organism being *S. enteritidis* phage type 8. The other 11 were negative for *Salmonella* and all 12 were negative for *Shigella*, enteropathic *Escherichia coli*, *Staphylococcus* and *Clostridium welchii*. Subsequent culture for virus was undertaken in 9 of these specimens on HeLa and on monkey kidney tissue but all were negative.

The *Salmonella* isolation was almost certainly an incidental finding. The clinical and epidemiological pattern of the outbreak did not fit with infection with this organism. None of the cases had antibiotic treatment and if a *Salmonella* had been responsible further isolations would have been certain. The other types of food poisoning can also be excluded in spite of the small number of specimens taken.

Table 4. *Results of water sampling (first outbreak)*

	Date	Number of coliforms per 100 ml. Tap over dishwasher	Tap over food-preparation sink
Public Health laboratory	15 May	180+	50
	23 May	180+	0
	24 May	3	0
	29 May	5	5
Water Company laboratory	20 May	0	1
	2 June	0	0

The lunch on 9 May consisted of fried or baked haddock, peas and chips, blackberry crumble and custard, all freshly cooked and served hot. Jugs of water were placed on the tables. A few people had biscuits and cheese instead of fish or instead of sweet. No single item was common to more than 75% of those who subsequently became ill. Even among the 13 non-resident staff, for whom this meal was the only one eaten in common with the other affected persons, there was no common item. No food remained for examination. Cheese was again served at supper that night and a sample of the same block was sent to the laboratory. No pathogenic bacteria were grown.

The water supply to Residence A is by rising mains from the Newcastle and Gateshead Water Company. This supply is of constantly proven excellence as far as bacteriological sampling is concerned. Specimens taken in the kitchen after running and after flaming the taps were negative for coliforms.

Samples were also taken without running and without flaming the taps. The results are summarized in Table 4.

Any significance of these results is very much open to question but the matter will be referred to later.

Faecal specimens were taken from all members of the kitchen staff, 4 of whom were affected and 10 of whom were not affected. All were negative for Shigella and food-poisoning organisms.

SECOND OUTBREAK

This affected 161 pupils aged 12 to 16 and 11 staff including two domestics at a secondary school between 9 January and 3 February 1971. One hundred and ten of the cases occurred between midday on 26 January and 9 p.m. on 27 January. The school had 343 pupils, 18 teaching or administrative staff and 7 domestic staff, a total of 368 persons. Of these 11 were absent at the relevant time leaving 357 exposed to infection.

Incidence

During December and January there were reports of gastro-intestinal infections in the area, and between 9 and 26 January 16 pupils were affected (Fig. 2). The main outbreak began in the late afternoon of 26 January and reached its peak

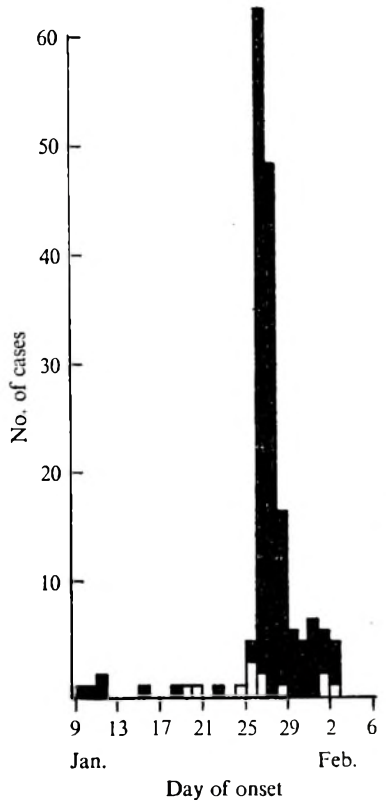

Fig. 2

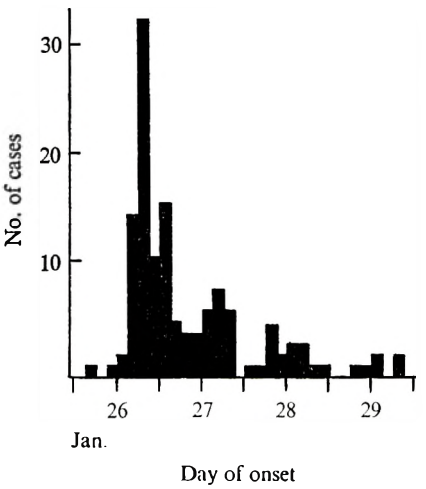

Fig. 3

Fig. 2. Onset of cases in second outbreak. □, Those not having school dinner; ■, those having school dinner.

Fig. 3. Onset of cases in second outbreak between 26 and 29 January, shown at 3-hourly intervals.

Table 5. *Incidence in relation to eating school dinner (second outbreak)*

	School dinner	No school dinner	Total
Affected between midday 26 January and midnight 27 January	109	1	110
Affected other times	51	11	62
Not affected	131	54	185
Total	291	66	357

$\chi^2 = 32{\cdot}97$; $P < 0{\cdot}0005$.

around 9 p.m. (Fig. 3) with a subsidiary peak at 5 a.m. on 27 January. Thereafter 46 cases occurred.

On 5 February a questionary was sent to all staff and parents. By interviewing those who had not completed the questionary information on all 357 persons was obtained.

The interesting feature of the outbreak is the preponderance of illness in those

Table 6. *Sex incidence in pupils (second outbreak)*

	M		F		Total	
Affected	94	52%	67	44%	161	48%
	58%		42%		100%	
Not affected	88	48%	84	56%	172	52%
	51%		49%		100%	
	182	100%	151	100%	333	100%
	55%		45%		100%	

Table 7. *Symptoms (second outbreak)*

	Staff		Students		Total	
	No.	%	No.	%	No.	%
Total numbers	11		161		172	
Nausea	9	81·8	116	72·0	125	72·7
Vomiting	8	72·7	110	68·3	118	68·6
Abdominal pain	7	63·6	96	59·6	103	59·9
Headache	5	45·4	67	41·6	72	41·9
Diarrhoea	7	63·6	49	30·4	58	33·7
Giddiness	3	27·3	29	18·0	32	18·6
Fever	2	18·2	20	12·4	22	12·8
Cramp	—		11	6·2	11	6·4
Aching legs or back	1		5		6	
Sweating	2		—		2	
Shivering	1		—		1	

Table 8. *Duration of symptoms (second outbreak)*

	No.	%
Up to 1 day	92	53
1–2 days	38	22
2–3 days	24	14
3–5 days	9	5
5–7 days	5	3
Over 7 days	4	3
Total	172	100

taking school meals on 26 and 27 January (Table 5). Eighty-two per cent of children have school dinners and the proportion affected outside the main outbreak is the same. However on 26 and 27 January 99% of those affected took school dinner.

In most of the outbreaks described girls have been more commonly affected than boys, but in this outbreak there was a slight preponderance of boys affected (Table 6).

Symptoms

A typical illness was nausea followed within an hour by vomiting and then colicky abdominal pain and diarrhoea. This was accompanied by headache. In

many cases vomiting occurred precipitately. Most of those affected were recovering within 24 hr. The symptoms of the 172 affected are given in Table 7.

Diarrhoea was a more common symptom among the staff (63 %) than among the pupils (30 %) as in the other outbreak. Comparison of the symptoms between those affected before and those during the main outbreak does not reveal any real differences. With the later cases there was a tendency for the illness to become milder and abdominal pain was the leading symptom with only 31 % having vomiting.

The duration of the symptoms is given on Table 8. Though the symptoms were often described as being severe the family doctor was consulted in only 16 cases (9 %).

In three instances two episodes of similar illness were mentioned with an interval of 2–3 weeks.

Family spread

As has been mentioned gastro-enteritis was fairly widespread in the district at the time. Of the 185 persons not affected 11 (6 %) had cases of diarrhoea or vomiting in the household. Of the 16 affected before the main outbreak 6 (38 %) had associated cases in the family.

From 26 January to 3 February 27 (17 %) stated that one or more members of the family were affected within seven days, with a total of 38 persons affected. Of the 27, in 3 the family case was before the case in the school, and in 5 at the same time. This leaves 19 (12 %) where a case occurred in the family which is most likely to be secondary to the main outbreak. (There were a number of households where more than one member attended the school and in these it has been assumed that during the main outbreak infection occurred at the school.)

Incubation period

The questionary did not ask for the hour at which the secondary case started but merely the day. Thus no accurate estimate can be made of the time interval in the 19 cases mentioned above. The interval between cases is as follows: 2 days, 11; 3 days, 1; 4 days, 3; 5 days, 4.

If it is assumed that the 109 children who developed symptoms on 26 and 27 January were affected at lunchtime on 25 January, then the mean incubation period is 37 hr. Subsequent cases at the school could either have had a longer incubation period or could be secondary to the main wave.

Investigations

Stool specimens were obtained from 7 affected persons and in none of these was there any growth of *Salmonella*, *Shigella*, *Staphylococcus*, *Clostridium welchii* or enteropathic *Escherichia coli*. No virus isolation was attempted. The number of specimens taken was small but our recent experience of an almost identical outbreak (*q.v.*) led us to diagnose epidemic vomiting at an early stage and not to seek too much help from the laboratory which at that time was experiencing difficulties.

About 280 school meals were served daily. The meal on Monday 25 January consisted of sausages, chips, carrots, chocolate sponge and custard. Water was available. All but 3 (97 %) of those affected on 26 and 27 January ate the sausage, all but 2 (98 %) had chips, 89 % had carrots, 85 % chocolate sponge or custard and 60 % had water.

Tuesday's meal was beef stew, potatoes, cabbage, date tart and custard. 4 persons affected on 26 January did not have this meal. Of those affected, 92 % had stew or potatoes, 75 % had cabbage and about 80 % sweet: 60 % had water.

It seems most unlikely that chips could transmit an infective agent so the sausages are the only item to come under suspicion as the vehicle of infection. Pupils and staff tended to blame the sausages. As well as complaining about the taste many said they were dark in colour. They had been cooked in the oven at gas mark 3 for $1\frac{1}{2}$ hr. Inquiry was made at the firm manufacturing the sausages. No person on the premises gave a history of gastro-enteritis. Sausages manufactured at the same time had been distributed to other schools and eaten without ill-effect. On balance it seemed unlikely that sausages were the vehicle of infection although the possibility could not be excluded.

The catering staff were all interviewed individually and asked about symptoms in themselves or their families before 26 January. One member had vomiting and abdominal pain on 13 January, an illness lasting for 3 hr., but there was no illness immediately before the main outbreak.

Water samples were taken from the kitchen taps without flaming or letting the water run but there were no coliforms per 100 ml.

DISCUSSION

The terms winter vomiting disease, non-bacterial gastro-enteritis and epidemic vomiting have all been used to describe similar outbreaks in which the illness itself was insignificant but large numbers of cases occurring simultaneously have caused concern. Major outbreaks have been described by Bradley (1943) and Webster (1953) with 200 and 300 cases respectively. Lesser outbreaks have been described by Miller & Raven (1936), Dummer (1953), Cumming & McEvedy (1969) and Adler & Zickl (1969) among others.

While the symptoms of these outbreaks have shown some variation the epidemiological pattern has been similar. Cases have been reported in the district with one or two in the institution about to be affected. There follows an explosive outbreak involving $\frac{1}{3}$ to $\frac{2}{3}$ of the institution's members. Finally there are a few secondary cases. The incubation period is from 1 to 3 days. The illness, often of sudden onset and occurring during the night, is usually afebrile and recovery is rapid. There are no sequelae. No causative organisms are found. Poisoning by a specific item of food can be excluded.

Outbreaks with similar clinical and epidemiological features have been traced to the ingestion of sewage-contaminated water (Lobel, Bisno, Goldfield & Prier, 1969; Public Health Laboratory Service, 1974); or to shellfish harvested from such water (Gunn & Rowlands, 1969; Public Health Laboratory Service, 1970; Ratzan,

Bryan & Krackow, 1969; Dismukes, Bisno, Katz & Johnson, 1969). The incidence in these outbreaks is usually particularly high, e.g. 38 out of 40 in the outbreak associated with oysters described by Gunn & Rowlands. Secondary person-to-person spread has occurred.

In other outbreaks such as those described by Zahorsky (1929), Gordon, Ingraham & Korns (1947) or Hopkins (1958) symptoms have been similar but the onset has been more gradual and person-to-person spread has probably been the principal mode of transmission.

In most outbreaks hysteria has been considered either as a primary cause or as a secondary feature. Most observers however have concluded that hysteria played an insignificant part. Typically the majority of cases occurred during the night and at home when the possibilities of mass suggestion are at a minimum. Outbreaks of fainting among schoolgirls (Pollock & Clayton, 1964; Moss & McEvedy, 1966) occurred typically during school hours. An outbreak of abdominal pain at a children's gala (Smith & Eastham, 1973) was thought on clinical and epidemiological grounds to be hysterical. These outbreaks have little in common with those described although Pollock & Clayton thought they were dealing with an infection.

Mode of spread

If the waterborne outbreaks are due to the same micro-organism as the other explosive outbreaks it would seem that one must look for a common source for these also. Poisoning by a single food can be excluded but is it possible for an infective agent to spread itself over several items of food? If someone in the kitchen of the institution concerned were transmitting the organism could they infect a working surface or a tap at the food preparation sink and spread subsequently take place to several items of food? Or could contaminated washing-up water lead to spread via crockery or cutlery?

These are purely theoretical possibilities but seem more likely than the respiratory spread postulated by some observers. Proof will need to await the identification of the causative organism. Recent experimental work (Paver, Caul, Ashley & Clarke, 1973; Kapikian *et al.* 1972) raises hope that this will soon occur.

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Serological comparison and haemagglutinating activity of *Mycoplasma dispar*

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SUMMARY

A comparison of twelve strains of *Mycoplasma dispar* by the metabolism inhibition and indirect haemagglutination tests has shown them to form a serologically homogeneous group of micro-organisms. The twelve strains vary in their haemagglutinating activity against erythrocytes from different animal species, and certain of the strains can be distinguished by the erythrocytes they agglutinate. Haemagglutination may thus provide a method by which certain strains can be typed. The erythrocyte receptor site does not appear to contain sialic acid and is not sensitive to proteolytic enzymes. On the mycoplasma cell two attachment sites have been demonstrated. One, by which it attaches to sheep and bovine erythrocytes, is a protein or contains a protein moiety. The chemical nature of the other attachment site, by which *M. dispar* attaches to rabbit erythrocytes, is unknown.

INTRODUCTION

Mycoplasma dispar was first isolated from pneumonic calf lungs (Gourlay, 1969; Gourlay & Leach, 1970) and subsequently from the respiratory tract of non-pneumonic calves (Thomas & Smith, 1972). Its role in calf pneumonia remains to be determined. However, the preliminary experiments of Gourlay & Thomas (1969) and St George, Horsfall & Sullivan (1973), who found lesions in the lungs of calves after endobronchial or intratracheal inoculation of broth cultures but not sterile broth, together with the observation that, of the mycoplasmas commonly found in the respiratory tract of calves in England, *M. dispar*, *M. bovirhinis*, *Acholeplasma laidlawii* and T-mycoplasma (Gourlay, Mackenzie & Cooper, 1970), only *M. dispar* had any cytopathic effect on bovine fetal tracheal organ cultures (Thomas & Howard, 1974) and the finding that certain strains of *M. dispar* cause clinical mastitis in cows experimentally inoculated (J. Brownlie, R. N. Gourlay & C. J. Howard – to be published) supports the hypothesis that this micro-organism is pathogenic for cattle, perhaps with a particular propensity for the respiratory tract.

Comparisons within certain mycoplasma species, e.g. *M. hominis* (Purcell *et al.* 1967) *M. gallisepticum* (Taylor-Robinson & Berry, 1969) and *M. pulmonis* (Forshaw & Fallon, 1972) have shown these species to be serologically heterogeneous and to contain various subtypes. No serological comparison of a group of *M. dispar*

strains has been made except for that of Gourlay & Leach (1970) who found that antiserum to strain 462/2 inhibited the growth on agar of 23 out of 36 strains examined. As little information is available on the serological heterogeneity or homogeneity of the species the first part of this study deals with this aspect of *M. dispar*.

Attachment to mucosal epithelium is considered to be the first step in the production of disease by many micro-organisms (Savage, 1972). The attachment of mycoplasmas to cells is well documented and differences between strains in their ability to attach to cells has been observed (Manchee & Taylor-Robinson, 1969*a*, *b*). The intimate association between mycoplasmas and cells has been proposed as a mechanism by which high local concentrations of toxic metabolites e.g. H_2O_2, are built up which may then cause cell damage (Sobeslavsky, Prescott & Chanock, 1968). In the second part of this paper the demonstration of attachment sites on *M. dispar* for erythrocytes is reported, and these attachment sites may represent the mechanism by which this micro-organism maintains itself in the bovine respiratory tract. Besides this, a study of haemagglutinating activity of *M. dispar* provides information on its surface structure and also forms the basis of a method useful for strain identification.

METHODS

Media

Mycoplasmas were grown in glucose calf-serum (GS) broth (Gourlay & Leach, 1970) containing ampicillin (Andrews, Leach, Gourlay & Howard, 1973) except when organisms were grown for injection into rabbits, in which case rabbit digest broth and rabbit serum, heated at 56° C. for 30 min. were substituted for Hartley's digest broth and fetal calf serum.

M. dispar strains

All strains were from the lungs of calves with pneumonia. In order that we should not select serologically similar strains the criteria used to identify organisms as *M. dispar*, in the first instance, were production of acid from glucose in GS broth and colony morphology. *M. dispar* forms atypical centreless colonies on primary isolation (Gourlay & Leach, 1970). The serological studies confirmed the identity of the strains used subsequently for haemagglutination. Antisera were prepared as described by Howard & Gourlay (1972). All strains were cloned by filtering broth cultures through 650 nm. Millipore filters, and propagation of single colonies on three successive occasions (Sub-committee on the Taxonomy of *Mycoplasmatales*, 1972).

Influenza A virus

Strain SIWG/1/57 was kindly provided by Dr A. S. Beare (Common Cold Unit, Salisbury) and stored at −70° C.

Serological tests

The metabolism inhibition test was performed according to Taylor-Robinson, Purcell, Wong & Chanock (1966). The indirect haemagglutination test was performed according to Herbert (1967) using a microtitre system (Cooke Engineering Co., Flow Laboratories, Irvine, Scotland), sonicated *M. dispar* as antigen and tanned sheep erythrocytes that had been stored in Alsevers solution (Wellcome Laboratories, Beckenham, Kent).

Haemagglutination

The various strains of *M. dispar* used were grown in GS broth at 37° C., harvested by centrifugation at 15,000 *g*, washed and resuspended in 0·15 M-NaCl and stored at −70° C. The optical density (OD) of mycoplasma suspensions at 500 nm. was measured with a 10 mm. light path cell in a Unicam SP 800 and haemagglutination titres are given for standard suspensions with an OD of 10.

Rabbit, rat, hamster, chicken, calf and guinea-pig red cells were collected and stored in Alsevers solution. Sheep and horse red cells in Alsevers solution were obtained from the Wellcome Laboratories and human group O citrated packed cells from the Regional Transfusion Centre, Oxford. All red cells were stored at 4° C. Before use they were washed three times with 0·15 M-NaCl and made up to the required concentration by volume.

Doubling dilutions of antigen were made in 25 µl. volumes of 0·15 M-NaCl using a microtitre system and U trays (Cooke Engineering Co.) and the red cells, 25 µl. of a 1 % v/v suspension, were added to the dilutions of antigens. The results were read after overnight incubation at 4° C. All haemagglutination titres are given as the reciprocal of the highest dilution of mycoplasma suspension that caused agglutination.

Treatment applied to erythrocytes

The methods followed were mainly those of Buckland & Tyrrell (1963). Receptor-destroying enzyme (Wellcome Laboratories, from *Vibrio cholerae*) diluted 1/4 in 0·15 M-NaCl was mixed with an equal volume of 5 % v/v erythrocytes. Equal volumes of the other reagents and 10 % v/v erythrocytes were incubated together. Trypsin (bovine pancreas type III) and chymotrypsin (bovine pancreas type I) were obtained from Sigma (Kingston upon Thames). Pronase (B.D.H., Poole) and erythrocytes were incubated together for 30 min. at 37° C. After the various treatments the cells were washed once with 0·15 M-NaCl, resuspended to give a 1 % suspension, and 25 µl. volumes were added to the doubling dilutions of the mycoplasma suspension.

Treatment applied to mycoplasmas

Equal volumes of mycoplasma suspension and the reagents listed in Table 5 were incubated together. The temperatures and times of incubation were as used by Buckland & Tyrrell (1963) for treating viruses. Pronase and mycoplasmas were incubated together at 37° C. for 30 min. After incubation the mycoplasmas were centrifuged and made up to their original volume.

Table 1. *Metabolism inhibition titres* of antisera to* M. dispar *strains*

Strain tested against:	Antiserum prepared against: F370	462/2	Vic12	Vic13	Gri 226	D2	D44	Vic7	Gri 250	Mmb 177	D52
F370	**2560**	1280	2560	640	2560	1280	2560	2560	1280	1280	1280
462/2	1280	**640**	640	640	160	80	160	40	80	160	320
Vic12	1280	640	**1280**	320	640	320	640	160	320	320	640
Vic13	1280	2560	1280	**640**	1280	640	1280	1280	640	640	640
Gri226	1280	640	1280	640	**320**	320	320	320	320	320	160
D2	2560	640	1280	640	1280	**1280**	2560	1280	1280	640	1280
D44	1280	1280	1280	1280	1280	1280	**1280**	1280	1280	640	640
Vic7	2560	1280	1280	1280	1280	1280	1280	**1280**	640	640	640
Gri250	1280	640	640	320	640	160	160	320	**160**	320	320
Mmb177	1280	1280	1280	640	640	1280	320	640	640	**640**	640
D52	2560	1280	1280	1280	1280	1280	1280	640	640	640	**1280**
Gri221	640	1280	640	160	320	160	80	160	160	160	160

* Reciprocal of highest dilution of antiserum inhibiting the production of acid from glucose.

Electron microscopy

M. dispar and erythrocytes were examined by electronmicroscopy after staining with ruthenium red (Springer & Roth, 1973) as previously described (Howard & Gourlay, 1974).

RESULTS

Serological comparison of twelve M. dispar *strains*

The results of the metabolism inhibition and indirect haemagglutination tests (Tables, 1, 2) confirmed that the strains, identified primarily on the basis of colony morphology and ability to produce acid from glucose, were *M. dispar*. The twelve strains studied form a serologically homogeneous group, only minor differences in antigenicity being indicated by the two tests. It was considered that these differences might be largely due to the variation in potencies of the different antisera, the state of growth of antigen in the metabolism inhibition test and the antigen concentration used to sensitize the red cells in the indirect haemagglutination test. Normal rabbit serum had titres of < 20 in the metabolism inhibition and indirect haemagglutination tests. No factor was observed analogous to that in rabbit sera which prevents the growth of human T-mycoplasmas (Howard & Gourlay, 1973).

Antisera were raised against organisms grown in medium containing rabbit digest broth and serum. However, in order to ensure that antibodies to bovine serum were not being detected in the indirect haemagglutination test, the dilutions of antisera and the final suspensions of tanned–sensitized sheep red cells were made in phosphate buffered saline containing 5% v/v fetal calf serum. Besides this, absorption of antiserum with bovine erythrocytes had no effect on the titre of antisera for sensitized red cells.

Table 2. *Titres* in indirect haemagglutination test of antisera to* M. dispar *strains*

Strain used as antigen	Antiserum prepared against: F370	462/2	Vic12	Vic13	Gri226	D2	D44	Vic7	Gri250	Mmb177	D52
F370	**5120**	5120	5120	5120	1280	2560	1280	640	640	640	320
462/2	10240	**5120**	320	1280	1280	320	640	80	160	640	320
Vic12	5120	2560	**10240**	20480	2560	2560	2560	1280	2560	640	1280
Vic13	10240	5120	2560	**5120**	5120	2560	640	640	320	2560	640
Gri226	20480	10240	5120	10240	**10240**	2560	2560	2560	1280	2560	1280
D2	20480	20480	5120	20480	5120	**20480**	10240	10240	5120	2560	1280
D44	10240	2560	1280	5120	1280	2560	**1280**	640	160	1280	320
Vic7	10240	5120	5120	10240	2560	5120	2560	**2560**	2560	1280	640
Gri250	40960	20480	10240	20480	10240	10240	10240	2560	**2560**	1280	1280
Mmb177	40960	40960	20480	40960	10240	10240	10240	10240	5120	**5120**	2560
D52	20480	5120	10240	20480	20480	5120	10240	10240	2560	2560	**2560**
Gri221	20480	20480	20480	20480	10240	5120	5120	10240	2560	1280	640

* Reciprocal of highest dilution of antiserum causing agglutination of sensitized erythrocytes.

Haemagglutination by M. dispar

Strains 462/2, Gri226, Gri221, F370, Mmb177 and Vic13 were tested for haemagglutinating activity against sheep, rat, rabbit, human, guinea-pig, hamster, chicken, horse and calf erythrocytes at 4°, 22°, and 37° C. There was little variation in agglutination titres at the different temperatures. However, the more concentrated mycoplasma suspensions caused haemolysis at 37° and 22° C. and subsequent tests were therefore performed at 4° C.

The growth of *M. dispar* strains F370 and Vic12 was studied over a 7-day period, the number of viable organisms per ml. measured (Gourlay & Leach, 1970) and the pH of the medium recorded. No difference was found in haemagglutinating activity of standard suspensions of these organisms measured after 2, 3, 4 and 7 days' growth.

The haemagglutinating activity of twelve strains for erythrocytes from nine animal species was measured. Samples from at least three animals of each species were tested on at least two occasions and the average haemagglutinating titre is shown in Table 3. The titre range was within one doubling dilution of the average given. The types of erythrocytes agglutinated by strain Mmb177 distinguished it from the eleven other strains examined. These other eleven strains showed a varied haemagglutinating activity. Strain Gri250 appeared the least active while, at the other end of the range strain Vic12 was the most active. Some of these eleven strains appeared rather similar but others could be distinguished on the basis of their haemagglutinating activity.

Effects of various treatments of red blood cells on their susceptibility to agglutination by M. dispar

Various treatments of red cells were carried out in an attempt to characterize the receptor site on the red cell surface. Strains F370 and Vic12 were chosen because they gave high haemagglutination titres and because their haemagglutinating

Table 3. *Haemagglutination titre* of* M. dispar *strains with erythrocytes from various animal species*

M. dispar strain	Source of erythrocytes: Rabbit	Sheep	Hamster	Calf	Horse	Chicken	Rat	Human	Guinea-pig
Vic12	32	16	16	8	16	16	8	16	8
D44	64	16	8	8	4	8	8	8	8
Vic13	32	4	4	8	2	2	1	2	1
F370	64	16	8	8	8	8	—	—	—
Vic7	32	8	4	4	1	1	1	1	1
D52	16	8	4	2	2	—	1	—	—
462/2	32	4	4	2	2	—	1	—	—
D2	32	4	4	1	1	1	—	—	—
Gri226	32	8	2	—	2	—	—	—	—
Gri221	32	2	—	—	—	—	—	—	—
Gri250	8	1	—	—	—	—	—	—	—
Mmb177	—	32	—	64	1	—	—	—	—

* Reciprocal of highest dilution of standard suspensions of *M. dispar* strains which agglutinated erythocytes.

Table 4. *Effects of various treatments of erythrocytes on the haemagglutination titre of* M. dispar *strain F*370

Treatment	Titre* against erythrocytes from: Rabbit	Calf	Sheep
Phosphate-buffered saline	64	16	32
Tris buffer	64	16	16
Pronase (0·02 %)	128	64	64
Trypsin (0·01 %)	128	64	32
Chymotrypsin (0·01 %)	64	32	32
Periodate (0·02 %)	32	16	8
Bisulphite (0·5 %)	64	16	32
RDE†	32	16	16

* Reciprocal of highest dilution of mycoplasma suspension causing haemagglutination.
† Receptor-destroying enzyme.

activity was representative of the majority of strains. The results obtained with both strains were essentially the same and those for strain F370 are given in Table 4. Erythrocytes from sheep, calves and rabbits were affected in essentially the same way by the reagents. Pronase and trypsin caused some red cells to become slightly more susceptible to agglutination. The other reagents had very little effect. The change with receptor-destroying enzyme was negligible compared with the change in susceptibility of erythrocytes to the stock suspension of influenza A virus, which had haemagglutination titres of < 4, 32 and 256 against calf, rabbit and sheep erythrocytes respectively before receptor-destroying-enzyme treatment and < 4 against all three types of erythrocytes subsequently.

Table 5. *Effects of various treatments of* Mycoplasma dispar *F370 on haemagglutinating activity*

Treatment	Titre* against erythrocytes from: Rabbit	Calf	Sheep
Phosphate-buffered saline	32	8	16
Tris buffer	64	8	16
Pronase (0·02 %)	64	< 2	2
Trypsin (0·01 %)	64	< 2	< 2
Chymotrypsin (0·01 %)	64	8	16
Periodate (0·1 %)	16	8	16
Bisulphite (1·0 %)	32	8	4

* Reciprocal of the highest dilution of mycoplasma suspension causing haemagglutination.

Effect of various treatments of mycoplasmas on haemagglutinating activity

In an attempt to characterize the attachment site on *M. dispar* two strains, F370 and Vic12, were treated with various compounds. The treatments affected both in essentially the same way and the results obtained for strain F370 are given in Table 5. The effect of treatments on the agglutination of bovine and sheep erythrocytes seemed similar but was different from the effect of the treatments on the agglutination of rabbit erythrocytes. Pronase and trypsin reduced the haemagglutination titre for bovine and sheep erythrocytes, but not for rabbit erythrocytes. On the other hand periodate, bisulphite and chymotrypsin had little effect.

Electron-microscopy

M. dispar strain F370 was incubated with sheep and rabbit erythrocytes at 4° C. for 1 hr. The mixture was then examined by electron-microscopy. The capsular material previously described by Howard & Gourlay (1974) can be seen outside the membrane (Plate 1). Fine threads of material seem to bridge the gap between the dense extramembranous material and the erythrocyte membrane.

DISCUSSION

The twelve strains examined formed a serologically homogeneous group and no serological heterogeneity was observed to the extent that exists amongst certain other mycoplasma species, e.g. *M. hominis* (Purcell *et al.* 1967), *M. pulmonis* (Forshaw & Fallon, 1972) and *M. gallisepticum* (Taylor-Robinson & Berry, 1969). The metabolism inhibition test was chosen because of its sensitivity and specificity, and indirect haemagglutination because of its sensitivity (Purcell, Chanock & Taylor-Robinson, 1969).

Considerable strain variation in haemagglutinating activity was observed. Strain Mmb177 appeared distinct from the other eleven strains examined. The other strains exhibited a graded haemagglutinating potency. Although serological methods do not appear to be useful for distinguishing strains haemagglutination may be. Haemagglutinating activity appears unrelated to serological structure for *M. dispar* as is the case with *M. gallisepticum* (Manchee & Taylor-Robinson, 1969*b*).

In contrast to certain other mycoplasmas (Manchee & Taylor-Robinson, 1968) neither the incubation temperature for the haemagglutination reaction nor the final pH of the growth medium appear to affect the haemagglutinating potency of *M. dispar*.

M. dispar strain 462/2 has been reported to have no haemagglutinating activity for bovine and guinea-pig erythrocytes (Erno & Stipkovits, 1973). The slight difference between the previously reported results and ours for this strain probably does no more than reflect the use of different methods. The variation observed between strains of the same species of mycoplasma emphasizes the necessity to study more than one representative before comparisons between different mycoplasma species are made.

The nature of the receptor site for mycoplasmas on erythrocytes and other cells and the nature of the attachment site on mycoplasmas have been investigated by other workers using haemagglutination and haemadsorption. However, it should be noted that haemagglutination and haemadsorption are not necessarily correlated (Manchee & Taylor-Robinson, 1968). Strains of *M. dispar* rarely form centres (Gourlay & Leach, 1970). It is therefore difficult to perform haemadsorption experiments because colonies wash off the agar.

M. gallisepticum, *M. synoviae* and *M. pneumoniae* have been shown to attach to various cells by means of sialic-acid-containing cell receptor sites (Gesner & Thomas, 1966; Manchee & Taylor-Robinson, 1969*a*, *b*). It has been suggested that in the case of *M. hominis* and *M. salivarium* the receptor sites are protein (Manchee & Taylor-Robinson, 1969*a*).

The experiments performed here to determine the nature of the receptor sites on erythrocytes for *M. dispar* indicate that they do not contain sialic acid since they are resistant to receptor-destroying enzyme (cf. influenza A virus). Furthermore the observation that bovine red cells are not agglutinated by influenza A virus but are agglutinated by *M. dispar* supports the hypothesis that *M. dispar* does not attach to a similar sialic-acid-containing erythrocyte receptor. The erythrocyte receptor site is resistant to bisulphite and periodate at the concentrations used. Treatment with pronase and trypsin perhaps makes erythrocytes slightly more susceptible to agglutination by *M. dispar*, possibly by altering the surface charge on the cell, although there is no relation between either the zeta potential or electrophoretic mobility of red cells from different animal species (values taken from Spector, 1956) and susceptibility to agglutination by *M. dispar*.

The results reported by Sobeslavsky *et al.* (1968) indicate that the attachment site of *M. pneumoniae* contains a lipid or glycerophosphate hapten. In contrast *M. salivarium* and *M. hominis* have been reported to have protein attachment sites (Manchee & Taylor-Robinson, 1969*a*; Hollingdale and Manchee, 1972). The attachment site on *M. dispar* for bovine and sheep erythrocytes is sensitive to pronase and trypsin. Since neither of these proteolytic enzymes destroys the mycoplasma attachment site for rabbit erythrocytes at least two attachment mechanisms must exist on the mycoplasma. The attachment site for bovine and sheep erythrocytes is therefore either a protein or contains a protein moiety. The chemical nature of the attachment site for rabbit erythrocytes is unknown.

Savage (1972) suggested that surface components similar to capsules may be involved in cell attachment by lactobacilli and the possibility exists that the capsule of *M. dispar* observed by electron-microscopy after staining with ruthenium red (Howard & Gourlay, 1974) may take part in cell attachment.

As has been stated by Manchee & Taylor-Robinson (1969*a*) the ability to attach to cells should not be regarded as necessarily indicating pathogenicity *in vivo*, but as a valuable property for an invading mycoplasma which might assist in the disease process. Thus the demonstration of attachment sites on *M. dispar* for erythrocytes may indicate the mechanism by which this micro-organism maintains itself in the bovine respiratory tract.

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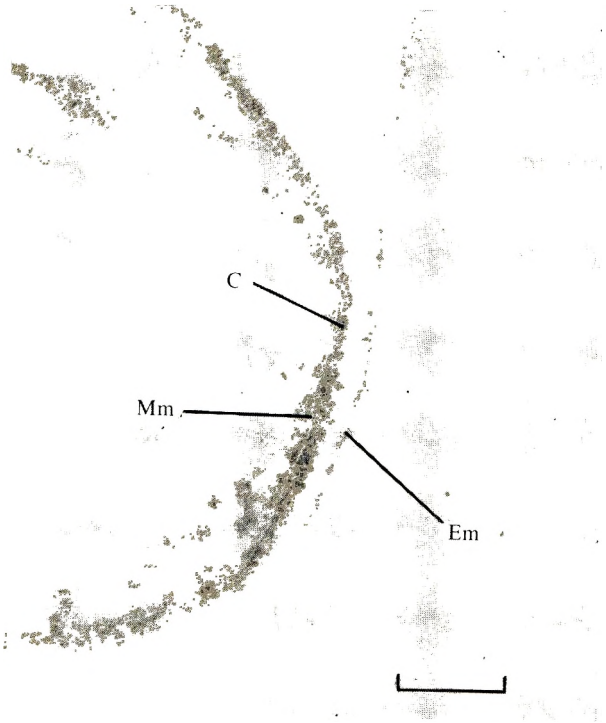
EXPLANATION OF PLATE

Electron-micrograph of *M. dispar* following incubation with rabbit erythrocytes. Mm, *M. dispar* membrane; Em, erythrocyte membrane; C, capsular material; bar marker, 100 nm.


C
Mm
Em

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Escherichia coli serotype distribution in man and animals

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SUMMARY

The serotype distributions of *Escherichia coli* isolated from animal faeces, human faeces and from meat have been compared. There were marked differences in serotype distribution in strains from man and animals. The meat strains generally resembled animal strains. The results suggest either that animal strains of *E. coli* are not reaching the general human population outside hospital to any great extent, or, if they do so, are failing to implant in the bowel.

INTRODUCTION

It has previously been suggested that animal strains of *Escherichia coli* regularly reach the human population via food (Cooke *et al.* 1970) and may implant in the human bowel (Shooter *et al.* 1970). In the present work, strains of *E. coli* previously isolated were typed using full O and H serotyping facilities. This was done in an attempt to determine whether animal and human strains of *E. coli* fall into distinct groups, or whether there is a serological overlap between *E. coli* strains from the two sources.

MATERIALS AND METHODS

Rectal swabs were obtained from cattle and pigs slaughtered in an abattoir and from chickens in a poultry packing station, as described by Cooke *et al.* (1970). Forty-eight specimens of meat were examined. These included beef, veal, poultry and pork. The meat was taken either from the hospital kitchen or from the homes of members of the laboratory staff. Preliminary work having shown that *E. coli* contamination of meat was confined to the surface, the meat was sampled by rubbing a swab over its surface. Faecal specimens were obtained from 55 persons; 7 from hospital staff and 48 from normal people outside the hospital. The specimens were plated on MacConkey's medium and 5 to 10 colonies of each colonial type examined. *E. coli* were identified as previously (Cooke, Ewins & Shooter, 1969). The strains of *E. coli* were serotyped using 150 O antisera and 51 H antisera

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Table 1. *Numbers of strains examined and serotyped from different specimens*

Source of strains	No. of specimens	No. of colonies examined	No. of different serotypes isolated from each source
Animals			
Cattle	38	274	139
Chickens	18	87	76
Pigs	11	65	60
Meat			
Beef and veal	25	52	36
Poultry	17	71	63
Pork	6	21	13
Persons			
Faeces	55	506	116

Table 2. *Distribution of O typable, non-typable and rough strains*

	Sources of strains						
	Animals			Meat			Humans
Types of strains	Cattle	Chickens	Pigs	Beef or veal	Poultry	Pork	Faeces
No. typable (150 O sera)	42	32	17	15	34	3	77
No. smooth but not O typable	66	34	33	18	24	4	14
No. O rough	31	10	10	3	5	6	25
Total	139	76	60	36	63	13	116

Table 3. *Distribution of H typable, non-typable and non-motile strains*

	Sources of strains						
	Animals			Meat			Humans
Types of strains	Cattle	Chickens	Pigs	Beef or veal	Poultry	Pork	Faeces
No. of typable (51 H sera)	126	57	41	25	43	7	71
No. motile but not typable	5	3	11	9	11	6	5
No. non-motile	8	16	8	2	9	0	40
Total	139	76	60	36	63	13	116

by tube agglutination based on methods previously described (Bettelheim & Taylor, 1969).

RESULTS

The number of specimens obtained, of *E. coli* examined and of the different serotypes found are given in Table 1.

The distribution of the typable, non-typable, rough and non-motile strains is given in Tables 2 and 3, and the distribution of common urinary infecting serotypes in Table 4.

Table 4. *Occurrence of common urinary serotypes in strains from the various sources*

	Sources of strains						
	Animals			Meat			Humans
Types of strains	Cattle	Chickens	Pigs	Beef or veal	Poultry	Pork	Faeces
No. of smooth strains	108	66	50	33	58	7	91
No. of common urinary serotypes (O1, O2, O4, O6, O7, O11, O18, O39 and O75)	4	3	1	8	9	1	26
% common urinary types of smooth strains	4	5	2	24	16	14	28

Table 5. *Source of O serotypes commonly associated with urinary tract infection*

Serotype	Source	Serotype	Source
O1:H6	cattle	O6:H1	humans
O1:H7	cattle, humans	O6:H16	chickens, poultry
O1:H45	poultry	O6:H45	poultry
O1:Hnt*	poultry	O6:H–	humans
O1:H–	chickens, humans	O7:H24	beef
O2:H5	beef	O7:H–	humans
O2:H6	humans	O11:H4	humans
O2:H7	cattle	O11:H16	poultry
O2:H8	cattle	O18:H7	humans
O2:H27	beef	O18:H14	cattle, beef, humans
O2:H–	beef, poultry	O18:H–	pigs, humans
O4:H1	humans	O39:Hnt*	humans
O4:H5	humans	O75:H55	humans
O4:H16	poultry	O75:H–	beef, pork, humans
O4:H42	poultry		
O4:H–	humans		

* nt, non-typable.

DISCUSSION

The number of strains which were O serotypable with the antisera used was much less among the animal population than among the human strains, with the meat strains resembling the animal strains. It was also noted that of the serotypes found, those commonly associated with urinary tract infections of humans and probably derived from the normal human faecal flora (Gruneberg, Leigh & Brumfitt, 1968) were found in relatively large numbers in the strains of human origin but rarely in the animal strains, with the meat strains falling between them (Table 4).

We have previously suggested that animal strains of *E. coli* reach the human bowel and can implant (Shooter *et al.* 1970). This is significant because of the widespread use of antibiotics in animal husbandry. It has been shown by Cooke, Hettiaratchy & Buck (1971) that the ingestion by volunteers of cultures of *E. coli*

of animal origin can result in these strains implanting in the bowel for long periods, although other workers have obtained rather different results (Williams Smith, 1969).

The difference found in the serotypes from animals and man with those from meat falling between them may reflect geographical variation in serotype distribution which has been shown to occur over quite small areas (Gruneberg & Bettelheim, 1969). All the human specimens came from people in the London area but it is possible that the results obtained with the abattoir specimens may reflect the distribution in animals from a limited number of environments. Nevertheless, the indications from this work are that the feed-through of *E. coli* from animals to man may be limited in the general population and that either serotypes from animals are generally not reaching the human population to any great extent, or they may not establish well in the human bowel. Most of the persons in this survey at the time of the study were eating mainly home-cooked food, that our own unpublished studies have shown usually to contain fewer *E. coli* than food served in hospitals and canteens (Shooter *et al.* 1971). The fact that a larger percentage of what are generally regarded as typically human serotypes were found in the meat strains would indicate contamination with strains of human origin during handling or selective survival of certain serotypes.

A difficulty in this work is the small amount of published material on the normal faecal flora of man and domestic animals. A large percentage of the results which are available have been obtained with limited numbers of antisera, because the authors were only looking for certain serotypes, such as the common enteropathogenic or urinary-tract-infecting serotypes. Unless a full range of O antisera is used and the numerous cross-reactions between various *E. coli* O types taken into account, an accurate interpretation of the results cannot be made because a number of O types might be grouped together.

The use of H antigen typing also indicated certain differences. Thus, on three occasions O6:H1 was isolated from human faeces but not from any of the other specimens. The only representatives of O group 6 found among the animal or meat strains were one strain of type O6:H16 from a chicken in the poultry packing station and two from chickens on arrival at the hospital kitchen.

It was also seen that for most O groups which are found in a number of different types of specimens, different H antigens appear associated with different sources. The sources of the O types which are commonly associated with urinary tract infections and their associated H antigens are shown in Table 5.

Three enteropathogenic serotypes were found: O86:H21, O114:H− and O128:H35. Two of these were found in the human faeces and the O114:H− serotype was found in a poultry packing station.

The results generally appear to indicate that there are obvious differences in serotype distribution of *E. coli* in man and animals. More work is required, particularly studying different geographical areas and *E. coli* serotype distribution in hospital patients.

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Food preferences of wild house-mice (*Mus musculus* L.)*

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SUMMARY

The relative acceptance of various plain foods by wild house-mice (*Mus musculus* L.) was compared in laboratory choice tests. The palatability of glycerine and six oils, each included at 5 % in pinhead oatmeal, was compared in a similar manner.

The most favoured food was found to be whole canary seed (*Phalaris canariensis*). Pinhead oatmeal and wheat were also comparatively well accepted. Glycerine, corn oil, arachis oil and mineral oil were more palatable than either olive, linseed or cod-liver oils.

The results of the choice tests are considered in relation to the use of poison baits for the control of free-living mice.

INTRODUCTION

The house-mouse (*M. musculus* L.) is cosmopolitan, occurring in cities and towns throughout the world. It is capable of causing significant economic losses, and adequate measures for its control are also important to prevent hazard to human health.

Most control work carried out against infestations of mice involves the close distribution of solid poison baits. Clearly the efficiency of this control method is dependent on having both effective poisons and attractive bait-bases at hand. The development of resistance to the hitherto effective anti-coagulant poisons (Rowe & Redfern, 1965) and the paucity and limitations of existing acute poisons has emphasized the need for more efficient poisons for the control of mice and stimulated a search for alternative chronic and acute acting compounds (Rowe, Greaves, Redfern & Martin, 1970). Apart from recent work by Norris (1973), however, less attention has been given to ensure that the most appropriate bait-bases are employed in poison treatments. For this reason a variety of different foods that are either currently used in Britain or that were considered to have potential use as bait-bases have been compared under laboratory conditions. Furthermore, since an oil is often incorporated as a sticker in formulating coarsely particled poison bait, the relative acceptability of different oils to mice was also investigated.

MATERIALS AND METHODS

The adult male and female mice that were used were the descendants of wild stock drawn originally from a corn-rick. Each animal was weighed, sexed and isolated in a metal test cage measuring $36 \times 30 \times 20$ cm. for at least 2 weeks before a choice test was begun. During this period it was provided with laboratory food (diet 41B), placed in a metal container that was situated centrally at the front of the cage, and with water *ad lib.* At the beginning of a test the food container and any spilled diet 41B were removed and two similar containers each holding a weighed amount of bait under test were positioned opposite each other at the front of the cage. The choice tests were conducted for 2 days using 5 mice and in some comparisons supplementary tests were run. The amount of bait eaten from each container was measured daily, the positions of the baits being reversed after 24 hr. to reduce the possible effects of place preference. A few mice were employed in more than one test, but in that event they were not offered a previously experienced bait.

The relative palatability of glycerine and six oils was compared in a similar manner. Each oil was included in a standard bait-base, pinhead oatmeal, at a concentration of 5%. In all tests the significance of the data was assessed using Student's '*t*' test.

RESULTS

Initial exploratory tests using a varied range of foods showed that some items were clearly preferred to others and only the most promising foods were closely examined in subsequent tests. Of the latter, whole canary seed (*Phalaris canariensis*), pinhead oatmeal and whole wheat were the most favoured. The results of choice tests conducted with each of these baits are shown in Tables 1, 2 and 3 respectively.

Overall, mice ate more whole canary seed than any other alternative food that was offered (Table 1), the relative difference in bait consumption reaching a significant level ($P < 0{\cdot}05$) in all but one of the 14 tests when flour was made available. Pinhead oatmeal was significantly less preferred than canary seed but it was consumed in greater amount than the 11 other baits (including flour) that were offered as a choice and in 8 cases significantly more pinhead oatmeal was eaten (Table 2). Although wheat was less preferred than either canary seed or pinhead oatmeal it was eaten in greater amount than 13 other baits, the difference in consumption being significant in 9 of the tests (Table 3).

Medium oatmeal is currently the most employed bait-base in poison treatments against mice in Britain but it was found to be poorly accepted in comparison with either canary seed, pinhead oatmeal or wheat. Rolled oats, another current but less frequently used bait-base, was also found to be less acceptable than either canary seed or pinhead oatmeal although it was significantly preferred to either flour, sausage rusk or maize meal. Flour, which is also used on occasion in poison treatments against mice, was significantly less preferred than pinhead oatmeal; more canary seed (17·5 g.) than flour (8·0 g.) was eaten but the difference in

Table 1. *The relative acceptance of whole canary seed and other baits by mice*

No. of mice tested	Amount of bait eaten (g.) Canary seed	Alternative		Difference (g.)
25	117·3	Wheat	30·5	+86·8*
25	114·9	Pinhead oatmeal	30·8	+84·1*
5	18·9	Medium oatmeal	8·5	+10·4*
5	24·0	Rolled oats	1·6	+22·4*
5	17·5	Flour	8·0	+ 9·5
5	29·6	Chick crumbs	2·2	+27·4*
10	42·2	Peanuts	4·9	+37·3*
5	33·1	Maize meal	4·8	+28·3*
5	24·7	Sunflower seed	3·2	+21·5*
5	16·9	Grass seed	7·9	+ 9·0*
5	24·8	Rape	0·2	+24·6*
5	24·4	Millet	1·6	+22·8*
5	23·2	Chocolate powder	4·1	+19·1*
10	49·9	Ground canary seed	15·6	+34·3*

* Significance of difference ($P < 0{\cdot}05$).

Table 2. *The relative acceptance of pinhead oatmeal and other baits by mice*

No. of mice tested	Amount of bait eaten (g.) Pinhead oatmeal	Alternative		Difference (g.)
25	107·7	Wheat	47·3	+60·4*
5	29·4	Rolled oats	1·3	+28·1*
5	27·3	Chick crumbs	0·6	+26·7*
5	11·6	Peanuts	8·6	+ 3·0
5	22·5	Sunflower seed	9·8	+12·7*
5	18·5	Grass seed	8·4	+10·1
5	6·8	Chocolate powder	3·9	+ 2·9
5	18·4	Sausage rusk	0·0	+18·4*
5	18·5	Medium oatmeal	0·4	+18·1*
5	23·2	Flour	1·8	+21·4*
5	21·4	'Bemax'	1·1	+20·3*

* Significance of difference ($P < 0{\cdot}05$).

consumption did not reach a significant level. In other tests flour was found to be significantly preferred to either fine oatmeal, sausage rusk, chocolate powder or diet 41B.

Two of the baits examined were presented in a different manner, canary seed in either whole or ground form and oatmeal as either pinhead (coarse) or medium grade. The larger particles of both baits were found to be preferred (Tables 1 and 2).

Only glycerine and corn oil were examined against each of the other 6 oils. The results of the tests (Table 4) indicated that glycerine, corn oil, arachis oil and mineral oil, in about that order, were more palatable to mice than either cod-liver, linseed or olive oils.

Table 3. *The relative acceptance of whole wheat and other baits by mice*

No. of mice tested	Amount of bait eaten (g.) Wheat	Alternative		Difference (g.)
5	12·8	Rolled oats	6·0	+ 6·8
5	15·6	Peanuts	10·1	+ 5·5
5	24·1	Maize meal	5·9	+18·2*
5	20·6	Sunflower seed	7·4	+13·2*
5	17·7	Grass seed	3·9	+13·8*
5	21·7	Chocolate powder	14·5	+ 7·2
5	22·2	Sausage rusk	0·3	+21·9*
5	19·9	Medium oatmeal	4·0	+15·9*
5	19·5	Flour	18·1	+ 1·4
5	21·7	'Bemax'	3·7	+18·0*
5	24·0	Sugar	0·8	+23·2*
5	34·6	Rice	1·1	+33·5*
5	26·1	Pearl barley	1·7	+24·4*

* Significance of difference ($P < 0{\cdot}05$).

Table 4. *The palatability of differently oiled pinhead oatmeal bait to mice*

Amount of oiled pinhead oatmeal bait eaten (g.)				Difference (g.)
Corn-oil	14·1	Cod-liver	5·8	+ 8·3*
	22·4	Linseed	4·4	+18·0*
	10·5	Arachis	10·3	+ 0·2
	21·6	Olive	1·9	+19·7*
	12·8	Mineral	7·0	+ 5·8
	6·4	Glycerine	11·3	− 4·9
Glycerine	22·0	Cod-liver	0·4	+21·6*
	19·8	Linseed	9·5	+10·3
	13·2	Arachis	8·9	+ 4·3
	17·4	Olive	1·0	+16·4*
	19·1	Mineral	6·6	+12·5
Arachis	16·8	Mineral	10·7	+ 6·1
Mineral	17·5	Linseed	9·9	+ 7·6
	18·6	Olive	5·9	+12·7
Cod-liver	8·3	Linseed	15·3	− 7·0

* Significance of difference ($P < 0{\cdot}05$).

DISCUSSION

Laboratory tests of the present kind can give, at best, a clear indication of those foods that are most likely to be of use as bait-bases in the field and, when a choice exists, the most appropriate form of a particular bait to employ. They cannot take into account, however, the numerous factors, environmental and behavioural, that can influence the acceptance by free-living mice of even the most promising bait-bases found in the laboratory. Southern (1954) pointed out the importance of such factors as the abundance and variety of alternative foods, the availability of cover and water, the effects of conditioning to staple foods and individual bait preference in determining the level of acceptance of bait-bases under field condi-

tions. He investigated a limited number of bait-bases in the laboratory and concluded that rolled oats with 20 % olive oil was the most promising candidate for field use. This bait was not invariably well accepted in field trials, however, and it was therefore advocated that a selection of likely bait-bases should be laid in mouse-infested areas for 1 or 2 days and the most favoured then selected for poisoning. Largely because of economic considerations this ideal approach is rarely, if ever, carried out in practice.

The results of the present laboratory work suggested that whole canary seed, which was not examined by Southern, might come nearer than rolled oats to fulfilling the need for an exceptionally palatable bait-base capable of diverting mice living in varied environments from existing food supplies. The acceptance of plain and of poison-treated whole canary seed has therefore been investigated in the field. In the evaluation of candidate rodenticides during the past 2 years, whole canary seed has been extensively used in census-baiting mice living in urban and rural premises where alternative food supplies were usually abundant. In each case the take of census bait indicated that the canary seed was well accepted. A similar conclusion was reached as a result of recent field trials when whole canary seed was used as the carrier for calciferol (vitamin D). In 6 treatments against mice 97–100 % control was obtained compared with 91–92 % control in 7 treatments using pinhead oatmeal (Rowe, Smith & Swinney, 1974). Although good control (97 %) was also achieved in a single trial using a mixed bait (rolled oats, pinhead oatmeal, wheat and canary seed) recommended by Norris (1973), examination of the baiting points showed that the canary seed was largely selected by the mice.

There is accruing field evidence therefore to support the laboratory findings that whole canary seed is attractive to mice. The attraction may be due in part to its shape or size or both. There is also evidence, tending to limit its general usefulness as a poison carrier, that mice discard the husks before consuming the seed. The success of the calciferol trials is considered to have been largely attributable to the use of impregnated poison bait. The calciferol was dissolved in corn oil and the solution thoroughly mixed with the canary seed. The mixture was then allowed to stand for 3 days to allow for the absorption of the poison solution. In contrast, ineffective control of a confined colony of mice was obtained when whole canary seed bait was merely surface-coated with the acute poison, gophacide (Thomson, 1971), using corn oil as the sticker. In the presence of an unattractive alternative food (diet 41B), a kill of only 10/15 was obtained in 7 days compared with a kill of 11/13 in 2 days using pinhead oatmeal treated with gophacide. This result occurred although the feeding data showed that more canary seed (16·0 g.) than pinhead-oatmeal-treated bait (5·1 g.) was eaten, indicating that some gophacide was discarded during husking. Thus it would seem possible to use whole canary seed as a poison carrier only with those poisons that can be dissolved in an acceptable solvent, e.g. water, glycerine, corn oil or arachis oil, to permit the preparation of impregnated bait.

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